

**SYSTEMIC AND GUT MUCOSAL IMMUNITY
TO
TISSUE TRANSGLUTAMINASE
IN
COELIAC DISEASE**

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DECLARATION

I declare that the work contained within this thesis is original and has been composed by me, with any exceptions clearly indicated. This research has been undertaken at the Gastrointestinal Laboratory, Department of Medical Sciences, University of Edinburgh, Western General Hospital, Edinburgh. This thesis has not been submitted for any other professional degree.

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SUMMARY

The diagnosis of coeliac disease (CD), a permanent gluten-sensitive enteropathy characterised by varying degrees of small bowel mucosal atrophy, requires histology. Serological tests are useful tools in selecting patients to undergo small intestinal biopsy. Serum anti-gliadin antibodies (AGA) lack disease specificity. Anti-endomysium (EmA) antibodies are more sensitive and specific, but are subjectively measured. The finding that the enzyme, tissue transglutaminase (tTG), is the autoantigen which interacts with EmA, has enabled the development of an enzyme-linked immunosorbent assay (ELISA) for measuring anti-tTG antibodies. The assay used guinea pig liver tTG as the substrate in the absence of a human recombinant form. In theory, the findings of the anti-tTG antibody and EmA assays should overlap, and the anti-tTG assay should be superior by providing an objective, semi-quantitative result which would be more practical for screening.

The aims of this research were to compare the sensitivity and specificity of serum and whole gut lavage fluid (WGLF) IgA AGA, IgA EmA and IgA anti-tTG antibodies in diagnosing untreated CD. The characteristics of untreated CD patients with a negative IgA EmA were also investigated. Serum and WGLF antibody concentrations were also compared with HLA DQ2 status and small bowel morphometry. Serum from 100 untreated CD patients, 25 dermatitis herpetiformis (DH) patients, 82 treated CD patients, 65 patients with normal intestinal histology, 245 gastrointestinal disease controls and 29 healthy volunteers were included. WGLF from 36 untreated CD and DH patients, 237 gastrointestinal disease controls and 13 volunteers were studied. Histological diagnosis was based on clinical presentation and not predominantly after the finding of positive serology.

Serum and WGLF IgA anti-tTG antibody concentrations were found to be significantly higher in untreated CD and DH patients compared with the control groups. Serum IgA anti-tTG correlated well with serum IgA EmA titres, declined following gluten withdrawal, but did not exactly overlap EmA findings. The sensitivities and specificities of the IgA anti-tTG, IgA EmA and IgA AGA assays were 78 and 97%, 86 and 100% and 60 and 86% respectively. WGLF IgA anti-tTG antibody concentrations were also found to be raised in 5 untreated CD patients with normal serum levels. The IgA EmA negative and positive CD patients were shown to have similar clinical characteristics, but the serum albumin was significantly lower in EmA negative patients. WGLF, but not serum, IgA anti-tTG antibody concentrations were significantly higher in HLA-DQ2 positive than HLA-DQ2 negative CD patients. The only association between antibody concentrations and intestinal morphometry was a weak positive correlation between serum IgA anti-tTG antibody concentrations and crypt depth.

This study demonstrated for the first time that IgA anti-tTG antibodies can be measured in gut secretions, and that serum and WGLF IgA anti-tTG antibody concentrations may be associated with intestinal morphometry and DQ2 status respectively. The finding of a raised WGLF IgA anti-tTG antibody concentration in a proportion of CD patients with normal serum levels supports the theory that intra-luminal IgA anti-tTG antibodies are locally produced. The use of a non-human tissue substrate in the IgA anti-tTG antibody ELISA may in part explain why the IgA EmA findings do not exactly overlap. As some untreated CD patients are EmA negative, serological methods cannot replace small intestinal biopsy for diagnosis.

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ABBREVIATIONS

ACR	Anti-calreticulin antibodies
AD	Anna Dahele
AL	Alaistair Lessels
APC	Antigen presenting cell
ARA	Anti-reticulin antibody
CD	Coeliac disease
CD4 or 8	Cluster of differentiation 4 or 8
CV	Coefficient of variation
Diarr	Infective/Idiopathic diarrhoea
DH	Dermatitis herpetiformis
EDTA	Ethylene diamine tetraacetic acid
EmA	Anti-endomysium antibody
ESPGAN	European Society of Paediatric Gastroenterology and Nutrition
F	Female
γ/δ	Gamma/delta
GFD	Gluten free diet
GI	Gastrointestinal
GTP	Guanosine triphosphate
Hcl	Hydrochloric acid
H&E	Haematoxylin and eosin
HiQC	High quality control

HLA	Human leucocyte antigen
HPLC	High performance liquid chromatography
HUC	Human umbilical cord
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICAM-1	Intercellular adhesion molecule 1
IDDM	Insulin-dependent diabetes mellitus
IEL	Intra-epithelial lymphocyte
Ig	Immunoglobulin
IL	Interleukin
JAB	Anti-jejunal antibody
LoQC	Low quality control
L/R	Lactulose/rhamnose
M	Male
MHC	Major histocompatibility complex
MW	Molecular weight
NaCl	Sodium chloride
ND	Not done
Non-CD	Non-coeliac disease control
NPV	Negative predictive value
OD	Optical density
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
PPV	Positive predictive value

PVA	partial villous atrophy
MO	Monkey oesophagus
QC	Quality control
ROC	Receiver operating characteristic
RT	Room temperature
SAPU	Scottish antibody production unit
SD	Standard deviation
SIgAD	Selective IgA deficiency
STVA	Subtotal villous atrophy
TCR	T cell receptor
TGF- β	Transforming growth factor- β
T _H	T helper
Tx CD	Treated coeliac disease
tTG	Tissue transglutaminase
TVA	Total villous atrophy
UC	Ulcerative colitis
Volunt	Healthy volunteers
WGL	Whole gut lavage
WGLF	Whole gut lavage fluid
χ^2	Chi-squared

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1. Anti-endomysium negative coeliac disease - Does additional serological testing help? British Society of Gastroenterology, Birmingham, March 2000.
2. Serum IgA anti-tissue transglutaminase in the diagnosis of coeliac disease. Caledonian Society Meeting, St. John's Hospital, Livingston, November 1999.
3. Organisation of the coeliac clinic and recent advances in serological testing. GI Staff Educational Meeting, Royal Infirmary of Edinburgh, June 1999.
4. Gut mucosal immunity to tissue transglutaminase. The Eighth International Coeliac Disease Conference, Naples, Italy, April 1999.
5. Recent advances in serological testing for coeliac disease. Head office staff and representatives of Nutricia in Bath, April 1999.
6. Serum and mucosal antibodies to tissue transglutaminase. The Travelling Gut Club, Western General Hospital, September 1998.

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CHAPTER 1: STUDY DESIGN & AIMS

1.1 Background

At the start of this project, there had only been one report identifying IgA and IgG anti-tTG antibodies in the serum of untreated CD patients (Dieterich et al. 1997). The ELISA method used in this study however, had only been assessed in 25 CD patients and 25 control subjects (healthy and disease). The spectrum of gastrointestinal (GI) diseases represented in the control group was only limited to inflammatory bowel disease (IBD) and alcoholic liver cirrhosis. In addition, the EmA status had not been determined in the control group and the proportion of EmA positive CD patients was not stated. This information is essential to objectively evaluate the sensitivity and specificity of the IgA anti-tTG antibody ELISA. These shortcomings were sufficient to warrant further evaluation in a larger group of untreated CD patients, GI disease controls and healthy volunteers. Other reports using serum have followed, but to date there are no studies investigating the presence of anti-tTG antibodies in gut mucosal secretions.

1.2 Study Design

This study prospectively investigated the presence of IgA and IgG anti-tTG antibodies, IgA EmA, and AGA in the serum and WGLF of histologically confirmed untreated CD patients, disease controls and healthy volunteers. In addition to the prospectively diagnosed CD patients, serum samples stored at -70°C from 72 patients with untreated CD and/or DH diagnosed between 1988 and 1996 were also analysed. The disease controls included patients with a wide range of GI disorders including Crohn's disease,

ulcerative colitis (UC), irritable bowel syndrome (IBS), idiopathic or infective diarrhoea, bile acid malabsorption, as well as GI polyps and malignancies. The numbers of patients studied in each group will be discussed in subsequent chapters.

The patients screened for CD had presented with symptoms such as diarrhoea, abdominal pain, weight loss or anaemia, or were in a high-risk group for CD such as insulin-dependent diabetes mellitus (IDDM) and DH. Gastrointestinal referrals were from general practitioners, haematologists, general physicians, diabetologists, dermatologists and the surgeons. A simple protocol for history taking, examination and investigation was designed to standardise the clinical and laboratory assessment of these patients. Baseline investigations performed in all CD patients, including serum electrolytes, glucose, calcium, thyroid function, full blood count, haematinics, and serum total immunoglobulins (Igs), were performed as part of the routine clinical chemistry laboratory service. Serum IgA AGA, IgG AGA and IgA EmA were performed using our already established in-house assays. Anti-reticulin antibodies (ARA) were measured in IgA EmA negative CD patients only by the microbiology laboratory. The IgA and IgG anti-tTG antibody concentrations were measured using our newly developed ELISAs for research purposes only. All patients with suspected small bowel malabsorption were asked to perform an intestinal permeability test using lactulose (L) and rhamnose (R). The L/R ratios were calculated from the urine samples submitted as part of the routine laboratory service.

Patients with clinical features of malabsorption (regardless of the serological results) and those with positive serological results were offered endoscopic duodenal biopsy with or without sedation. Three to four distal duodenal biopsies were obtained from all patients using 37K biopsy forceps (SB-37K-1, Olympus Endotherapy). Histological assessment of the duodenal biopsies was performed routinely by the pathology department on haematoxylin and eosin (H&E) stained tissue sections. In the

prospectively diagnosed group of CD patients, histological assessment was also performed using the Marsh grading system (Marsh, 1992). Patients with histologically confirmed CD were asked to perform a WGL for research purposes before starting a gluten-free diet (GFD), so that the concentrations of intra-luminal antibodies and proteins could be measured. Follow-up serology was obtained at visits to the newly formed coeliac clinic to monitor the change in antibody concentrations after dietary treatment. This study was designed by the late Professor Ferguson who obtained funding from the Scottish Office and ethical approval from the Medicine and Oncology Subcommittee of Lothian Ethics in Research Committee.

1.3 Aims & Objectives

The primary aims of the study were as follows:

1. To develop in-house IgA and IgG anti-tTG antibody ELISAs, using a guinea pig liver tTG substrate, for the measurement of anti-tTG antibodies in serum and WGLF.
2. To compare the sensitivity and specificity of IgA anti-tTG antibodies with IgA EmA, and IgA and IgG AGA in diagnosing untreated CD patients in order to determine if the anti-tTG antibody assay is superior to the existing serological methods.
3. To investigate the change in concentration of serum IgA anti-tTG antibodies following treatment with a GFD.
4. To determine the prevalence of IgA EmA negative untreated CD patients and to investigate the clinical characteristics and serological findings in this sub-set of patients.
5. To investigate the origin of IgA anti-tTG antibodies in WGLF and to compare antibody concentrations in serum and WGLF.

6. To compare serum and WGLF IgA anti-tTG antibody, IgA EmA and IgA AGA concentrations with HLA DQ2 status, histological severity, morphometric measurements and intestinal permeability in CD patients.

1.4 Outline of Thesis

The following chapter gives a historical perspective on the pathogenesis of CD and reviews the performance of the available serological methods. The third chapter outlines the stages in the development of the IgA and IgG anti-tTG antibody ELISAs and the next chapter gives the detailed methodology of all of the tests applied in this thesis. The fifth chapter explores the use of the IgA anti-tTG antibodies in the diagnosis and monitoring of CD patients. The specificity of the assay is determined by the inclusion of a large number of disease controls, non-coeliac patients and healthy volunteers. The following chapter describes the clinical, genetic, serological and histological characteristics of IgA EmA negative CD patients. In chapter 7, serum and WGLF IgA anti-tTG antibodies are measured in untreated CD patients and controls. In chapter 8, the relationship between serological tests and DQ2 status, histological severity, morphometry and intestinal permeability is determined. In the final chapter, the role of the IgA anti-tTG antibodies in the diagnosis, pathogenesis and future treatment of CD will be explored.

CHAPTER 2: GENERAL INTRODUCTION

2.1 Historical Perspective of Coeliac Disease

Coeliac disease (CD) is a permanent gluten-sensitive enteropathy characterised by small bowel mucosal atrophy. The characteristic manifestations of CD were first recognised by a London physician, Samuel Gee in 1887 (Gee, 1888). Later in 1908, an American Professor of Pharmacology and Therapeutics, Dr. O.A. Herter, published a similar description of a wasting disease with steatorrhoea in children, and hence for some time, the condition was known as “Gee-Herter’s disease” (Corazza et al. 1988; Walker-Smith, 1988). Although Gee acknowledged that diet was a key part of treatment, he did not recognise the specific contribution of cereals.

Throughout the history of CD, dietary manipulation has been the mainstay in therapy. Early dietary therapies were associated with considerable mortality. These included the low complex carbohydrate diet, the banana diet, the low fat diet, the Fanconi diet (fruit and vegetables), the beef steak cure and the milk diet (van Berge-Henegouwen and Mulder, 1993). It was not until 1932 that a Dutch Paediatrician, Professor Willem-Karel Dicke, at the University of the Hague, started to make the link between wheat and CD. He made a causal association during the wartime “winter of starvation”, when the delivery of normal foods such as bread was very limited. He observed that CD patients clinically improved when their meals were prepared from rice or potato flour instead of wheat flour. His later thesis in 1950, studying the response of CD children to a wheat-free diet, clearly identified wheat flour to be responsible for the anorexia and steatorrhoea seen in CD (Dicke, 1950). The basis of treatment of CD with a GFD has remained essentially unchanged over the years.

2.2 Evolving Theories on Gluten Toxicity & the Pathogenesis of Coeliac Disease

Gluten was discovered by Jacopo Bartolomeo Beccari, Professor of Chemistry at the University of Bologna in 1728 (Corazza et al. 1988). He separated two substances from wheat flour, a water-soluble component called starch, and a water-insoluble protein component called gluten. Dicke's collaboration with two biochemists, Professor Dolf Weijers and Jan van de Kamer, led to further progress in identifying the specific causal agent. The biochemists had a technique for estimating faecal fat excretion, which was an important objective technique to confirm Dicke's clinical observations. The group tested a wide range of cereals, including wheat, rice, oats, potato, corn and rye flour, and only wheat and rye flours were found to be injurious (Dicke, 1953). Incremental addition of gluten into the diet led to anorexia, weight loss and steatorrhoea which was objectively measured by variations in faecal fat content as well as by clinical response (van de Kamer et al. 1953; Dicke, 1953). The protein fraction of wheat, gliadin, proved to be the responsible factor. When gliadin was chemically broken down to examine its various fractions, it was noted to have an abundance of glutamine residues (43%) but a low content of lysine, methionine and other amino acids (van de Kamer and Weijers, 1955). The investigation of the harmful effect of gliadin was concentrated on its unusually high glutamine content.

Van de Kamer *et al.* also showed that feeding with protein-bound glutamine, and not free glutamine, exerted an adverse clinical or biochemical response (van de Kamer and Weijers, 1955). In addition, they subjected gliadin to a process of acid deamidation, such that 90% of the glutamine residues were changed to glutamic acid and ammonia, while preserving the protein structure of gliadin. This deamidated gliadin, when substituted for regular gliadin in CD children, led to a clinical improvement in both cases. These experiments showed that free glutamine and acid-deamidated gliadin were

not toxic. Others working closely with Dicke supported his findings (Anderson et al. 1952).

The alcohol-soluble fraction, known as prolamins, in wheat (gliadin), rye (secalins), barley (hordeins) and oats (avenins) are responsible for the toxicity (Godkin and Jewell, 1998). The amide content (glutamine-Q and proline-P) ranges from 40% in gliadin to 13% in avenins, and correlates with their relative abilities to cause exacerbations in CD. There is a marked sequence homology between the corresponding prolamins of wheat, rye and barley, but not with oats. Gliadins can be separated by electrophoresis into 4 groups designated α -, β -, γ - and ω - gliadins, but α -gliadin (particularly the A-gliadin fraction) is thought to be the most active (Anderson et al. 2000). The main disease-precipitating prolamins are known to contain repetitive sequences based on PSQQ or SPQQ, (serine-S) (Marsh, 1992). The finding that gliadin shares an amino acid sequence that has about 80% homology with the Elb coat protein of the human adenovirus 12, has raised the possibility that a cross-reactive epitope may be involved in the pathogenesis of CD (Kagnoff et al. 1987). However, subsequent work has failed to identify any adenovirus 12 inserts in the DNA extracts from CD mucosa (Carter et al. 1989).

Several theories have evolved speculating the pathogenesis of CD. The “missing peptidase” hypothesis, coined by Frazer *et al.* in 1956, proposed that CD was the result of an inborn error of metabolism with a deficiency of a specific, unidentified mucosal peptidase which led to the accumulation of indigestible, toxic gluten peptides (Frazer, 1956). The theory arose following the observation that CD patients relapsed after being fed peptic tryptic-pancreatic digested gluten, but if this was incubated first with normal pig intestinal mucosa, the resultant mixture was no longer toxic (Frazer, 1956). It was thus thought that the relevant enzymes were present in the pig intestinal wall and not in the intestinal secretions. Although other reports supported this theory, none were able

to demonstrate a specific peptidase deficiency in the small intestinal mucosa of CD patients (Bronstein et al. 1966; Messer et al. 1961). Only one study has suggested the identity of the missing peptidase. This report found that gliadin was rendered non-toxic by exhaustive digestion with crude papain (a commercial product prepared from the latex of the unripe papaya) (Messer et al. 1964). The authors proposed that glutamine cyclotransferase might be the enzyme in crude papain, but warned that the enzyme may be different in normal small intestine.

The “lectin” hypothesis proposed that gliadin may act as a lectin (Weiser and Douglas, 1976; Unsworth et al. 1987), which are carbohydrate binding proteins present in most plants, especially cereals, potatoes and beans (Freed, 1999). Gliadin can bind to glycoproteins expressed in the epithelial cell membrane (Weiser and Douglas, 1976) or in reticulin (Unsworth et al. 1987), rendering them immunogenic. Currently, the most widely accepted hypothesis is the “immunological” theory, which proposes that there is a local hypersensitivity reaction to gluten or to a gluten peptide (Marsh et al. 1993; Biagi et al. 1999b). An immunological mechanism has been supported by the beneficial response of adult CD patients to corticosteroid treatment (Lepore, 1958), the presence of antibodies to gliadin in the serum of CD patients (Taylor et al. 1961), the infiltration of the small intestinal mucosa in CD with inflammatory cells (Marsh et al. 1993), and the strong association with particular human leucocyte antigen (HLA) genes on the short arm of chromosome 6 (Sollid and Thorsby, 1993). There is strong evidence of a genetic susceptibility, as there is a high prevalence rate of between 10-20%, in the first-degree relatives of CD patients (Maki et al. 1991). As there is only a 70% concordance rate among monozygotic twins, it suggests that environmental factors are also important (Sollid and Thorsby, 1993).

2.3 Systemic & Mucosal Immunology

2.3.1 General Aspects

The systemic and mucosal immune systems have evolved to protect the host from pathogens such as infectious agents. The cells that mediate immunity includes lymphocytes, which recognise antigens on the surface of pathogens, and phagocytes, which engulf, internalise and degrade pathogens. Antigens are molecules that are recognised by receptors on lymphocytes. There are two lymphocyte populations, B and T cells, both of which are derived from the bone marrow. B cells usually recognise intact antigen molecules and produce specific antibodies directed against them (humoral immunity). T cells recognise antigen fragments on the surface of antigen presenting cells and are involved in the process of cell-mediated immunity. Malfunction of the immune system may lead to hypersensitivity, autoimmune diseases, or immunodeficiency.

B cells are genetically programmed to encode an antigen-specific surface receptor. After specific antigen recognition, B cells multiply and differentiate into plasma cells, which produce large amount of soluble receptor molecules known as antibodies. Antibodies all have a basic structure comprising two identical light and two identical heavy chains which are linked together by disulphide bonds. Differences in the heavy chains give rise to five Ig classes (IgG, IgA, IgM, IgD and IgE) and to subclass distinctions. There are four human IgG subclasses (IgG1-4), two IgA subclasses (IgA1-2), but none have been described for IgM, IgD or IgE. All Ig molecules have one region of the molecule that is concerned with binding to antigen, the "binding site". The other region mediates "effector functions" such as the binding of Ig to various cells of the immune system, to some phagocytic cells and to complement components (Turner, 1998).

IgG is the major Ig in normal human serum accounting for 70-75% of the total Ig pool. It consists of a single 4-chain molecule with a molecular weight (MW) of 146, 000, and is distributed evenly between the intravascular and extravascular pools. IgM accounts for about 10% of the Ig pool and exists as a pentameric molecule with a MW of approximately 970, 000. IgM is mainly confined to the intravascular pool. IgA represents about 15-20% of the human serum Ig pool and more than 80% of it occurs as a monomer of the 4-chain unit. IgA is the predominant Ig in mucosal secretions such as saliva, milk and GI, tracheo-bronchial and genitourinary secretions (Turner, 1998).

There are several different types of T cells. T helper cells (T_H cells) interact with B cells helping them to divide, differentiate and produce antibody. They also interact with mononuclear phagocytes and help them to destroy intracellular pathogens. Another group, the cytotoxic T cells kill host cells that have become infected by viruses or other intracellular pathogens. Antigen recognition occurs via the antigen-specific T cell receptor (TCR), which is a heterodimer comprising α and β , or γ and δ peptide chains which form α/β , or γ/δ dimers respectively. T cells recognise antigens only when they are presented on the surface of “antigen presenting cells” (APCs) bearing specific HLA molecules, which are involved in the rejection of foreign or non-self tissues.

The human HLA complex is organised into three regions (I, II and III) which reflect the way that these molecules permit alloantibodies to bind and destroy leukocytes. The class I and II gene products have the same overall structure. The class I molecule comprises a glycosylated heavy chain linked to a serum polypeptide, β_2 -microglobulin. The class II molecules, DR, DQ and DP, are heterodimers of heavy (α) and light (β) glycoprotein chains which have the same overall structures. Lymphocytes display specific surface markers which are classified according to the “cluster of differentiation” (CD) terminology. The CD3 marker is expressed by mature lymphocytes and is closely associated with the TCR. The CD8 or CD4 markers on the T cell surface interact with

the HLA class I and II molecules respectively. T cell activation occurs after the TCR recognises a part of an antigen (epitope) bound to the HLA class I and II molecules, expressed on the surface of APCs (Sawyerr et al. 1993). T cell effector functions result from direct cell-cell interactions, or via the release of soluble glycoproteins called cytokines, including interleukin (IL), interferon (IFN) and tumour necrosis factor (TNF) molecules, which signal other cells.

There are two different profiles of cytokine production of CD4⁺ T_H cells (T_H1 and T_H2). The T_H1 cells are involved in cell-mediated inflammatory reactions including inflammatory and delayed hypersensitivity reactions. The typical cytokines produced include IFN γ , TNF- β and interleukin-2 (IL-2). In contrast, the T_H2 cells encourage the production of antibodies, especially IgE, and are associated with the regulation of allergic responses. The cytokines produced include IL-4-6, IL-9, IL-10 and IL-13. Cytokines from T_H1 cells inhibit the actions of T_H2 cells and *vice versa*, therefore immune responses tend to settle into either a T_H1-type or a T_H2-type of response.

2.3.2 Systemic Immunity

The major source of circulating IgA in humans is from the bone marrow. Smaller contributions to serum IgA are probably made by the spleen and peripheral lymph nodes, including the tonsils. Although almost equal quantities of IgA and IgG are produced and secreted into the circulation each day, the serum IgA concentration is much lower than IgG as it has a shorter half-life (3-6 days and 21 days respectively) (Conley and Delacroix, 1987). As IgA has a short half-life and is unable to initiate inflammatory reactions, it is able to play the role of "housekeeper", removing relatively harmless antigens from the circulation that have leaked in from the GI or respiratory tracts (Stokes et al. 1980). Indeed, IgA directed against antigens encountered at the mucosal surfaces can frequently be detected in serum. This may be because B cells exposed to antigens at mucosal surfaces may migrate to sites of antibody production,

and also because antigens may penetrate the mucosal surfaces where they are able to stimulate the humoral immune system (Conley and Delacroix, 1987).

2.3.3 Gut Mucosal Immunity

The intestinal immune system has the largest collection of immune cells in the body. It appears to have evolved to allow selective absorption and exclusion of the vast array of intra-luminal antigens to which it is continuously exposed. Some fed antigens induce a state of "oral tolerance" which is a state of hyporesponsiveness to later parenteral administration of the same antigen. Intra-luminal antigens, entering the small intestinal mucosa via specialised cells in the Peyer's patches, activate T and B lymphocytes. Activated T and B cells migrate via the lymphatics to mesenteric lymph nodes and some cells pass from here into the systemic circulation via the thoracic duct. The cells are then re-circulated to the intestine and are disseminated in the lamina propria throughout the length of the intestine (Sawyerr et al. 1993).

The intestinal mucosa surface epithelium contains another population of lymphocytes, the intra-epithelial lymphocytes (IELs) which are interspersed among the single layer of epithelial cells. It is thought that local differentiation, mitotic expansion and migration of lamina propria T cells may contribute to the size of the IEL pool (Marsh, 1992). There are no B cells in the normal IEL compartment (Sawyerr et al. 1993). In the normal intestinal mucosa, only about 10% of IELs express γ/δ TCR (Viney and MacDonald, 1990) and about 90% of IELs are CD3+, of which 80% are also CD8+ (Sawyerr et al. 1993). In contrast, in the lamina propria, more than 99% of the T cells express α/β TCR and about 80% of these cells are CD4+ (Sawyerr et al. 1993).

The presence of Igs in human intestinal secretions was recognised a decade later than serum IgA (Holman et al. 1959). The origin of these antibodies, by local production in the gut mucosa, was supported by the demonstration of Ig producing cells in the lamina

propria of human jejunal mucosa (Crabbe et al. 1965). Secretory IgA, which may be either subclass IgA₁ or IgA₂, exists as a dimer made up of two units of IgA linked by a J chain and has a protein known as the secretory component wrapped around it. The secretory component aids the transport of IgA and makes secretory IgA more resistant to proteolysis than serum IgA or IgG (Tomasi, 1992). The secretory component is present not only within the crypt epithelium, but also in the surface epithelium where its concentration rises with increasing villous atrophy (Colombel et al. 1990; Wood et al. 1987). It is therefore not thought to be a rate-limiting factor in Ig secretion into the jejunal lumen in CD (Brandtzaeg et al. 1988).

As the gut mucosal immune system has separate and distinct cell types and Ig isotypes, effector functions, and immunoregulation from the systemic immune system, serum IgA does not directly reflect IgA produced at mucosal surfaces (Ferguson et al. 1994). Mucosal-derived IgA has been shown to play a significant role in the body's defence against allergens and micro-organisms (Tomasi et al. 1965). A fundamental difference between the two systems is that in the gut, there are almost equal quantities of IgA₁ and IgA₂ proximally (Engstrom et al. 1992; Volta et al. 1990), with progressively more IgA₂ distally, whereas in the serum IgA₁ predominates (88-90%) (Brandtzaeg et al. 1989).

There is strong evidence that serum IgA in humans does not make significant contributions to IgA in GI fluids. This was demonstrated by a study showing that only 2% of jejunal fluid contained plasma derived IgA with the rest, mainly secretory IgA, being locally produced within the intestinal lamina propria (Jonard et al. 1984). In this study, the proportion of IgA₂ in secretions was identical to the percentage of IgA₂ positive plasma cells in jejunal biopsies from the same individuals. It also appears that the gut is not the main source of serum Igs as children acquire adult levels of mucosal IgA by the age of one year, but do not acquire adult levels of serum IgA until about age

12 (Conley and Delacroix, 1987). In addition, mucosal IgA is unlikely to be the source of serum IgA in humans as the thoracic duct lymph is not enriched for IgA and contains about the same concentration of IgA in serum, with the same monomer-polymer distribution (Kaartinen et al. 1978; Brown et al. 1982). These findings suggest that serum and intestinal IgA may be from different origins, and that intra-luminal antibodies are produced locally in the gut mucosa.

2.3.4 Gut Immune-Mediated Mechanisms in Coeliac Disease

The gluten-sensitive inflammation involves the full thickness of the intestinal mucosa, but the lamina propria appears to be the major site affected (Marsh, 1992). Indeed, MHC class II-expressing macrophages in the lamina propria have been shown to be activated and able to present gliadin peptides to CD4⁺ T lymphocytes (Devery et al. 1990). In addition, activated macrophages also lead to the up-regulation of IL-2 receptors by CD4⁺ lamina propria T cells (Brandtzaeg et al. 1988). Pro-inflammatory cytokines, produced from activated lamina propria T cells lead to the chemotaxis of neutrophils, mast cells and basophils. Activation of these cell types causes further production of inflammatory mediators, which leads to complement activation, microvascular hyperpermeability and oedema of the lamina propria. The cytokine profile seen in CD is predominantly in keeping with a T_H1-type response (Lahat et al. 1999). It is unknown why a similar pattern of cell-mediated reactions seen in small bowel Crohn's disease do not lead to the same kind of architectural remodelling seen in CD (Marsh, 1992).

In untreated CD, the proportion of IELs expressing γ/δ TCR is increased to over 30% and this remains high in treated CD even after the mucosal morphology has returned to normal (Arranz et al. 1994; Viney and MacDonald, 1990). In normal subjects, only about 10% of IELs express γ/δ TCR. Although the functions of IELs remain unknown, it has been proposed that these cells may become cytotoxic after the presentation of

gliadin peptides by epithelial cells, as the γ/δ TCRs are not HLA class restricted (Viney and MacDonald, 1990). However, evidence is available that IELs are not directly involved in cytotoxicity. Intercellular adhesion molecule-1 (ICAM-1), which is required by all lymphoid cells to mediate cytotoxicity (Marsh, 1992), is completely absent from the epithelium, but is abundant in the lamina propria (Sturgess et al. 1982). In addition, raised IELs are not exclusive to CD, and the density may also be increased in the absence of any change in the mucosal architecture (Marsh, 1992).

CD has a strong genetic predisposition and was first found to be associated with the HLA class I molecule B8 (Falchuk et al. 1972; Stokes et al. 1972). Stronger associations with the class II molecules DR3 (Keuning et al. 1976), DR7 (Sollid and Thorsby, 1993), DQ2 (Tosi et al. 1983) and DQ8 (Spurkland et al. 1997) were later reported. In European countries, the HLA phenotype shows a South-North gradient, with HLA DR3 and DR7 more common in the North and the South respectively (Greco et al. 1998). The DR3-DQ2 haplotype showed the strongest association with CD (Sollid and Thorsby, 1993). The combination of two HLA-DQ alleles, HLA-DQ α 1*0501 and HLA-DQ β 1*0201, is found in about 90-95% of British CD patients (Sollid and Thorsby, 1993; Marsh et al. 1993; Balas et al. 1997) and most DH patients (Balas et al. 1997). A minority of patients carry the HLA-DR4 associated DQ β 1*0302 haplotype, also known as HLA-DQ8 (Pena et al. 1998). Non-HLA genes on chromosome 6, such as the TNF genes are also thought to influence the severity of the disease (Pena et al. 1998).

It was suggested that characterisation of the prolamins recognised by DQ2 (α 1*0501, β 1*0201)-restricted T cell clones may reveal the identity of the injurious prolamins (Sollid and Thorsby, 1993). HLA class II molecules bind and endocytose prolamins, which are then degraded in an intracellular endosomal/lysosomal compartment where the resulting peptides associate with new

class II molecules (van de Wal et al. 2000). These HLA class II-peptide complexes are then transported to the cell surface for recognition by peptide specific CD4+ T cells. T_H cells then give help to antigen-specific B cells to help them produce specific antibodies.

Many groups have shown that jejunal lamina propria plasma cells are increased in untreated CD (IgA>IgM>IgG) (Wood et al. 1987; Colombel et al. 1990; Jonard et al. 1984), and in DH (Lancaster-Smith et al. 1974). The number of plasma cells also decline following treatment with a GFD (Wood et al. 1987; Scott et al. 1984; Lancaster-Smith et al. 1974). The presence of Igs in gut secretions from patients with CD or Crohn's disease may partly be due to an increased intestinal permeability allowing the passage of luminal antigens into the submucosa and triggering an immune response. The finding of a high proportion of secretory IgA (20-36%) in the serum of untreated CD patients raises the possibility that the production of secretory IgA could exceed the capacity of epithelial transport, and result in an over-spill from the intestine into the circulation (Volta et al. 1990). The absence of raised levels of circulating secretory IgA in normal individuals may be explained if there was rapid clearance from the serum by the liver as occurs in rats (Conley and Delacroix, 1987). Others have shown that IgA AGA is predominantly in the dimeric form in serum (not linked to secretory component), but is in the polymeric form (attached to secretory component) in jejunal fluid (Colombel et al. 1990). Workers from our laboratory have previously demonstrated a marked dissociation between antibody responses to gliadin in serum and gut secretions in CD (O'Mahony et al. 1991).

Another contributing factor to the CD lesion comes from the changes in the microcirculation where the formation of microthrombi (as a result of activation of the endothelium and platelets by inflammatory mediators), may lead to ischaemia and loss of surface epithelium. The failure of the CD mucosa to regenerate may be because of a failure of neovascularization, although the factors inhibiting this process are unknown.

The epithelium and enterocytes themselves do not appear to have a direct pathogenic role (Marsh, 1992).

The classical description of the CD lesion is of a flat avillous mucosal surface. It is recognised however, that this represents an advanced lesion and that other earlier changes may also warrant a diagnosis of CD. Marsh has described five main lesions (types 0-4) which may be associated with gluten-sensitive enteropathy (Marsh, 1992). The pre-infiltrative (type 0) lesion, seen only in DH patients, is indistinguishable from normal, but the intestinal secretions of these patients contain high concentrations of anti-gliadin antibodies (AGA). This finding may be an indicator of latent CD, however it is uncertain if such jejunal lesions would progress in time to a more severe lesion. The infiltrative (type 1) lesion comprises a normal mucosal architecture in which there is a marked infiltration of IELs. This lesion has been shown to be inducible by gluten (Leigh et al. 1985; Ferguson et al. 1987). The hyperplastic (type 2) lesion also has a dense infiltration of IELs, but with the addition of enlarged crypts. The destructive (type 3) lesion reflects the typical flat mucosal lesion associated with CD, and occurs in most symptomatic patients although it may also be asymptomatic, "silent CD". The hypoplastic/atrophic (type 4) lesion represents the end-stage of gluten sensitivity in which the mucosa is chronically unresponsive to gluten withdrawal. The types 1, 2 and 3 lesions are not specific to untreated CD, and may also be seen in giardiasis, tropical sprue, graft versus host disease and in milk or soya allergy. Typical type 0 and type 3 lesions are shown in **(Figures 2.1 & 2.2)**.



Figure 2.1: Marsh type 0 lesion with preserved villous architecture

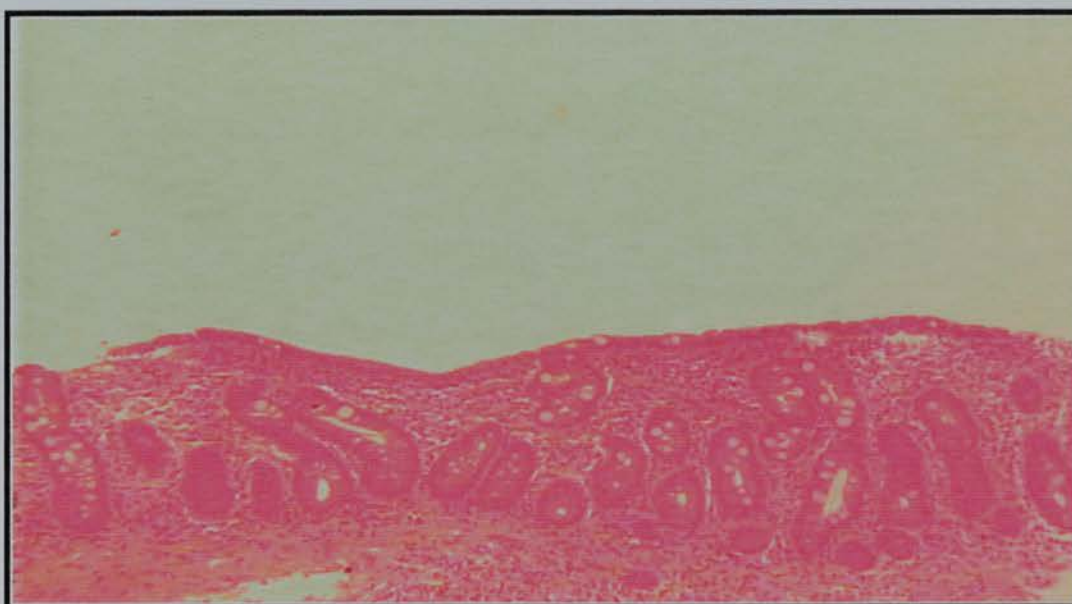


Figure 2.2: Marsh type 3 (destructive) lesion with total loss of villi

2.3.5 Assessment of Gut Mucosal Immunity in Coeliac Disease

The concept of a “common mucosal immune system” proposes that the specific induction of the mucosal immune system at a site of immunisation could lead to the simultaneous production of antibodies in various distant secretions (Mestecky, 1987). The implication of this theory would be that secretory IgA responses resulting from antigen presentation at one mucosal site, might be monitored successfully by measuring secretory IgA at a distant mucosal site. If this concept were true, then IgA levels in saliva would in theory reflect those in the intestine. Unfortunately, although saliva is a readily accessible mucosal secretion, the measurement of specific antibody concentrations in saliva has not been shown to reflect gut mucosal immunity (Engstrom et al. 1992; Kelly et al. 1991; O'Mahony et al. 1991; Labrooy et al. 1980).

The study of gut mucosal immunity has been complicated by the more difficult access to intestinal tissues and secretions (O'Mahony et al. 1990), difficulties in accurately and reproducibly measuring antibody content, and the presence of bacteria and bacterial proteases which can damage the secretory IgA (Challacombe, 1995). The use of protease inhibitors, standardised collection techniques, storage conditions and the development of sensitive ELISAs have overcome some of these problems (Challacombe, 1995). Small intestinal secretions have been collected by aspiration using a segmental perfusion technique (Colombel et al. 1990; Lavo et al. 1992; Engstrom et al. 1992), via the Crosby capsule (O'Mahony et al. 1991; Volta et al. 1990; Arranz and Ferguson, 1993) or endoscope at the time of small intestinal biopsy (Kelly et al. 1991). The use of the per-oral isotonic saline lavage technique (Svennerholm et al. 1982) is limited by its contra-indication in patients with cardiac, renal and liver disease (O'Mahony et al. 1990). Although secretory IgA can be measured in faeces, immunological tests on faeces and on saline extracts have been shown to be misleading (Ferguson et al. 1995).

In contrast, the whole gut lavage (WGL) technique, described in 1988 by Gaspari *et al.* (Gaspari *et al.* 1988), is a safe, effective and non-invasive method of investigating mucosal immunity. The method is essentially a whole gut perfusion system, with the first clear effluent termed WGLF. The method has the advantage of being conveniently incorporated into clinical investigation as it is often used as a bowel preparation for endoscopic or barium studies or for surgery (Ferguson and Mwantembe, 1995). It has been demonstrated that early faecally contaminated specimens contain negligible amounts of Igs, but once clear, there is very little variation in Ig content (O'Mahony *et al.* 1990). The loss of Igs in WGLF due to proteolysis by intra-luminal bacterial proteases, can be reduced by treating specimens with protease inhibitors (Gaspari *et al.* 1988). IgA is the most abundant Ig in WGLF and most of it is in the secretory form.

The WGLF has been widely used to assess intestinal immunity (Ferguson and Mwantembe, 1995; O'Mahony *et al.* 1990; O'Mahony *et al.* 1991), gut inflammation and protein loss (Choudari *et al.* 1993; Brydon *et al.* 1993; O'Mahony *et al.* 1991), and the presence of malignant cells (Rosman *et al.* 1994) or blood (Brydon and Ferguson, 1992) within the gut lumen. Specific antibodies to food antigens (gliadin, betalactoglobulin and ovalbumin) (O'Mahony *et al.* 1991), and to cholera toxin B subunit (Gaspari *et al.* 1988) can be measured in WGLF. Early studies of food antibodies in intestinal fluids of CD patients were based on relatively insensitive precipitin tests that were unable to distinguish between the various Ig classes (Ferguson and Carswell, 1972). More recent studies have shown raised concentrations of IgA and IgM AGA antibodies in WGLF and jejunal aspirate (O'Mahony *et al.* 1991; Lavo *et al.* 1992), with a positive correlation between WGLF and jejunal aspirate but not with saliva (O'Mahony *et al.* 1991). There was a significant positive correlation between serum and jejunal IgA AGA in CD patients (Lavo *et al.* 1992). A "coeliac-like" intestinal antibody pattern has also been described as a marker of latent gluten sensitive enteropathy (Arranz and Ferguson, 1993).

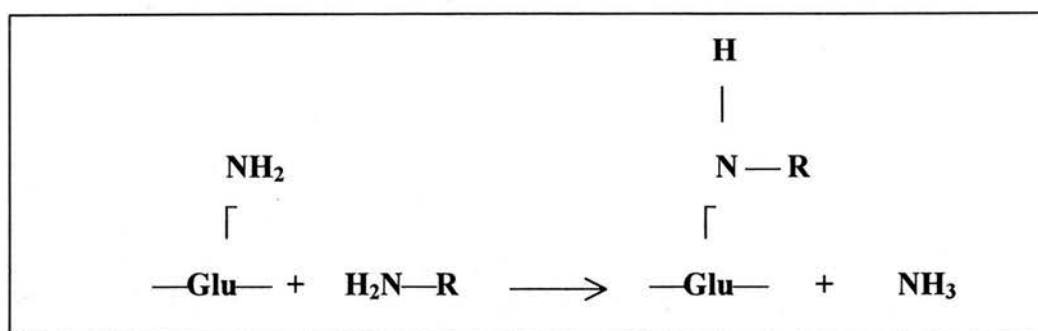
2.4 Tissue Transglutaminase

2.4.1 Tissue Transglutaminase Distribution, Structure & Function

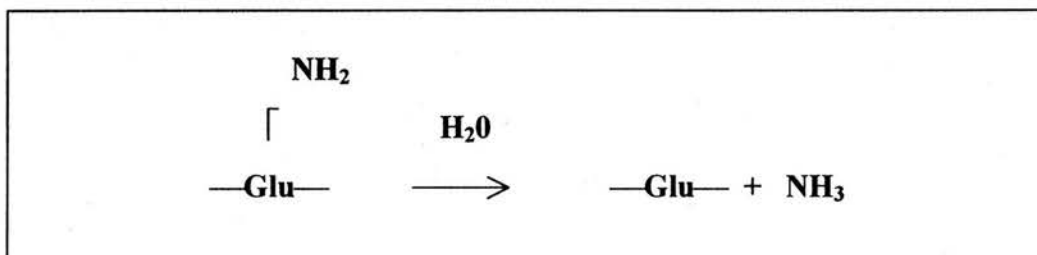
The term transglutaminase (TG) was first introduced in 1959 (Mycek et al. 1959), but its existence had been known about much earlier (Duckert et al. 1960). The TGs are widely distributed in various organs, tissues and body fluids and are mainly distinguishable from each other by their physical properties and locations in the body (Bruce et al. 1985; Ichinose et al. 1990). The TGs have been classified into three distinct groups based on their physical, chemical, immunological and enzymatic properties (Chung, 1999):

1. Serum TG (factor XIII) of plasma, platelets, placenta and uterus
2. Tissue TG (tTG) of all organs - in order of decreasing activity in the guinea pig: (liver, spleen, kidney, adrenal, heart, lung, intestine, testis, brain, pancreas, muscle)
3. Hair follicle TG

Tissue TG contains no disulfide bonds or carbohydrate and hence, unlike serum TG, it appears to exist as a single chain consisting of 690 amino acid residues with a MW of 75-85 000 (Folk, 1983). It contains 16-18 sulphhydryl groups of which only one is essential for its catalytic activity. The mechanism of action is similar for all of these enzymes (Chung, 1999). They all catalyse the formation of iso-peptide bonds between the γ -carboxyl groups of glutamine residues in one polypeptide chain with the ϵ -amino groups of lysine residues in another forming an ϵ -(γ -glutamyl)-lysine bond and ammonia (Folk, 1972). The calcium-dependent reaction is shown as follows:



In the absence of protein-bound lysine residues, TG catalyses hydrolysis at the carboxamide group of peptide-bound glutamine (a process called deamidation) to give peptide-bound glutamic acid and ammonia as follows:



Unlike the peptidoglutaminases which also catalyse reactions at the carboxamide group of peptide-bound glutamine residues, TGs cannot act on free glutamine residues (Folk, 1972). The TGs have a very limited donor substrate (glutamine-rich) specificity but an exceptionally broad specificity for primary amine acceptors (peptide-bound lysines or polyamines) (Folk, 1983). The mechanism of action of the TGs enables these enzymes to perform post-translational modification of protein molecules (Folk, 1972; Folk, 1983).

Calcium is essential for enzyme activation of all forms of TG, except possibly for hair follicle TG (Ichinose et al. 1990). The interaction between TG and calcium ions causes a conformational change in the enzyme that is essential for its catalytic activity, without

producing gross changes in the size or shape of the molecule (Folk, 1983). Guinea pig liver tTG is activated at an optimal calcium concentration of 10-15 mmol/l and pH of between 8.0 and 8.5 (Bruce et al. 1985). Tissue TG also binds guanosine triphosphate (GTP) inducing a conformational change, which inhibits its enzymatic activity (Greenberg et al. 1991). However, as calcium ions inhibit GTP binding, it suggests that local concentrations of GTP and calcium could regulate intracellular TG activity *in vivo* (Greenberg et al. 1991).

Tissue TG is restricted to the cytoplasm of cells, as the DNA sequence lacks an NH₂ - terminal hydrophobic leader sequence, which is required for its secretion (Greenberg et al. 1991). The mechanism of the release of serum TG into the cytoplasm is not known. The majority of tTG (about 97%) has been located within the cytoplasm of human umbilical vein endothelial cells and vascular smooth muscle cells (Chowdhury et al. 1997). Guinea pig liver has been chosen as the source of the enzyme for studies as it is a rich source of the enzyme due to the expression of tTG by liver sinusoidal endothelium (Folk, 1972; Greenberg et al. 1991). The enzyme is obtained from the cytoplasm of cells by fractionating homogenates of fresh guinea pig liver tissue (Folk, 1972). The amino acid sequence of tTG is highly conserved among the species suggesting they have evolved from a common ancestral gene (Greenberg et al. 1991). Of 51 residues, 49 are identical in the active site region and there is more than an overall 80% sequence homology between human and guinea pig liver tTG (Ichinose et al. 1990; Greenberg et al. 1991). The commercially available guinea pig liver tTG (Sigma) is not pure and may vary from batch to batch (Maki, 1997a). A human recombinant form of the enzyme is not yet commercially available.

The TGs have been implicated in many physiological settings. Activated serum TG is the final enzyme in the coagulation cascade (Gladner and Nossal, 1983). It has an important role in the stabilisation of the fibrin clot by causing polymerisation of the α -

chains of fibrin, producing a tough insoluble fibrin clot that is resistant to plasmin degradation (Gladner and Nossal, 1983). The intracellular functions of tTG are still not fully understood. It is thought to have an important role in the regulation of cell growth, differentiation and apoptosis (Fesus et al. 1987). Enzyme levels are higher in involuting cells where it produces extensive cross-linking of cytoplasmic and membrane proteins, thereby maintaining cellular integrity until the formation and phagocytosis of the apoptotic bodies (Fesus et al. 1987). It also has a role in the formation of a cross-linked cytomatrix (Chowdhury et al. 1997).

In the extracellular matrix, tTG has an important role in wound healing. The extracellular matrix is a dynamic network of adhesive proteins functioning not only as a barrier to cellular migration, but also has important metabolic and regulatory functions (Upchurch et al. 1991). Tissue TG acts as the first line of defence after injury by attaching to fibronectin immediately after cell rupture (Upchurch et al. 1987). The mechanism of release of tTG from injured cells appears to be via passive leakage from ruptured cells, as tTG is not found in the extracellular matrix under normal conditions, but appears rapidly after wounding (Upchurch et al. 1991). In the presence of calcium, tTG then has cross-linking activity with extracellular matrix proteins (fibronectin, collagen II, V, XI, and procollagen II) producing bonds which are stable and resistant to proteolysis, thereby increasing the resistance of tissues to chemical, enzymatic, and physical degradation (Upchurch et al. 1991; Upchurch et al. 1987). Fibronectin is susceptible to the action of tTG and serum TG owing to its high content of glutamine residues (Upchurch et al. 1991). Cross-linked fibronectin promotes wound healing by providing a preferred surface for the migration of epithelial and fibroblast cells (Upchurch et al. 1987).

Tissue TG has also been implicated in a number of disease states. In Alzheimer's disease, tTG concentrations are increased in the prefrontal cortex and it is thought to

contribute to the formation of insoluble neurofibrillary tangles by cross-linking soluble tau proteins, which are an excellent substrate for the enzyme (Appelt and Balin, 1997). TTG gene expression is increased during hepatic injury and fibrosis suggesting a role in fibrogenesis (Mirza et al. 1997). The role of tTG in the pathogenesis of CD will be discussed in (section 2.4.3).

2.4.2 Tissue Transglutaminase & Autoantigens in Coeliac Disease

The observation that serum antibodies from untreated CD patients interact with jejunal mucosa (Seah et al. 1971b; Hallstrom, 1989; Karpatai et al. 1990), has suggested the presence of circulating autoantibodies directed against self-antigens. The identification of autoantigens in CD has eluded researchers for many years. Early workers proposed that the autoantigen was a non-collagenous reticulin component isolated from kidney tissue, but it later transpired that reticulin was not a single entity (Pras and Glynn, 1973). Later, using human foetal lung tissue, others identified and purified six human non-collagenous protein molecules which specifically bound to serum IgA anti-reticulin antibodies (ARA) and EmA from untreated CD patients (Maki et al. 1991). This group subsequently showed that fibroblasts synthesised and secreted these autoantigen molecules (Marttinen and Maki, 1993).

Another candidate autoantigen was calreticulin, a high capacity calcium binding protein (Karska et al. 1995). Others showed that human umbilical vein endothelial cells contained an intracellular autoantigen that bound IgA from untreated CD patients, and that this autoantigen was antigenically similar to that found in reticulin and endomysium (Whelan et al. 1996). The spectrum of autoantibodies in CD was thought to be wider than that covered by EmA and ARA as it was shown that antigens isolated from sheep lung and monkey intestines were able to absorb EmA and ARA, but the rat liver component did not bind (Borner et al. 1996). Although the autoantigen(s) in CD are

thought to be highly conserved in rodents and primates, this group did not investigate the interaction with human tissues.

A recent landmark study unequivocally demonstrated that tTG was the autoantigen with which EmA interact in CD (Dieterich *et al.* 1997). An immunohistochemical technique was used on cell cultures of human fibrosarcoma (cell line HT 1080) cells and human embryonal fibroblasts (W138) as well as human hepatocarcinoma (Hep1 and Hep 2) cells, to demonstrate the expression of the autoantigen with high-titre sera from untreated CD patients. Subsequent immunoprecipitation of the supernatant and cell lysates with the IgA fraction from serum samples of CD patients, yielded a protein identified as fibronectin and a single protein band with a MW of 85 kDa respectively. Immunoprecipitation of cell lysates occurred only with the 25 CD serum samples, but with none of the 25 control samples (from healthy adults, patients with ulcerative colitis, Crohn's disease, or Sjogren's syndrome). Fibronectin was not thought to be the primary target as some immunoprecipitation occurred in controls, but instead may have precipitated non-specifically or in association with the 85 kDa autoantigen. Following further analysis and using amino-terminal sequence analysis, the 85 kDa protein was identified as tTG. The co-precipitation of tTG with fibronectin observed was thought to be due to the known interaction of these two proteins (Chowdhury *et al.* 1997).

In order to further confirm that tTG was the autoantigen in CD, Dieterich *et al.* performed indirect immunofluorescence with high-titre CD serum samples on monkey oesophagus with or without prior pre-incubation of the sera with Sigma tTG (Dieterich *et al.* 1997). Untreated CD serum showed the characteristic honeycomb endomysial staining pattern, but pre-treatment with Sigma tTG nearly completely abolished endomysial immunofluorescence. This demonstrated that tTG represented the predominant, if not sole, endomysial autoantigen considered characteristic for CD. Others have noted that Dieterich *et al.* did not demonstrate that the 85 kDa protein possessed tTG activity, nor did they show a positive immunoblot with monoclonal tTG

antibody (Maki, 1997a). Despite these potential pitfalls, there has been growing evidence to support that tTG is indeed the long sought after autoantigen in CD.

2.4.3 Tissue Transglutaminase in the Pathogenesis of Coeliac Disease

The possible pathogenetic role of tTG in CD has been speculated upon for many years. The enzyme was first implicated in the pathogenesis of CD by a group suggesting that tTG may facilitate gluten binding to membrane components (Peters and Bjarnason, 1984). They suggested that it might not be that a “peptidase” was missing, but might be excessively expressed in CD. The following year, the same group were the first to demonstrate tTG enzyme activity in human jejunal mucosa using a radiometric assay, and to show that the enzyme kinetics were similar to that in other tissues and to guinea pig liver tTG (Bruce et al. 1985). They also showed that the jejunal mucosal tTG activity was significantly higher in untreated CD patients than in IBD patients or controls. Treated CD patients had a higher mucosal tTG activity than controls, but lower activity than untreated CD patients. Gliadin was shown to be an excellent substrate for the enzyme owing to its high glutamine content (40% of glutaminy residues), a necessary requirement for tTG activity. Indeed, when comparing tTG activity using a variety of protein substrates (gliadin, collagen, ovalbumin, elastin and zein), they found the highest tTG activity was with gliadin, which was comparable to that of the standard substrate, dimethylcasein.

Using a rodent model, it was later shown that intestinal tTG activity was predominantly submucosal (>85%) as opposed to epithelial (10%) (Patel et al. 1985; D'Argenio et al. 1988), and that tTG activity progressively increased from the duodenum to the terminal ileum (D'Argenio et al. 1988). Indeed tTG activity was demonstrated throughout the length of the rat GI tract with the highest activity found in the oesophagus and gastric antrum (Patel et al. 1985). An inverse relationship between mucosal tTG and serum

TG activity was found, such that while there was an increase in mucosal tTG in untreated CD patients, the serum TG activity was reduced (D'Argenio et al. 1989). The increased mucosal activity was thought to reflect the proliferative response in mucosal cells. These findings were significant because they demonstrated that tTG had a high affinity for gliadin, and that the tTG-gliadin interaction was unlikely to occur at the gut mucosal surface as initially thought. A submucosal localisation of tTG would not in itself exclude the role of tTG in the pathogenesis of CD, since even in treated CD, the mucosa remains abnormally permeable to macromolecules despite histological remission (Bjarnason and Peters, 1984; Bjarnason et al. 1983).

Speculation followed that dietary gliadin, now known to have a high affinity for tTG, unmasked "cryptic epitopes" leading to the production of ARA and EmA (Maki, 1996). It was proposed that damage or hyperpermeability of the intestinal epithelium, either due to direct gluten toxicity or other irritants, could trigger the release of intracellular tTG (Dieterich et al. 1997). Subsequent crosslinking of dietary gliadin could result in the formation of gliadin-gliadin or gliadin-tTG complexes. The creation of antigenic neoepitopes could then initiate an immune response in genetically susceptible individuals, directed both to gliadin and tTG.

An alternative mechanism for the production of anti-tTG antibodies was that in the normal state, B cells specific for self-antigens exist, but they only produce antibodies when given help by T cells (Sollid et al. 1997). In addition, auto-reactive T cells are normally deleted in the thymus, and hence anti-tTG specific T cells should not be present in normal subjects. HLA DQ2-restricted gliadin-specific T_H cells, which are present in small intestinal biopsies of CD patients but not in non-coeliac controls (Molberg et al. 1997), can provide such help (Sollid et al. 1997). It was suggested that when gliadin and tTG are linked, these T_H cells could provide help for tTG-specific B cells. Following dietary gluten withdrawal, T cell help for the anti-tTG specific B cells

would cease and the concentrations of anti-tTG antibodies would decline. It is unlikely that tTG-specific T cells are responsible for providing the help, as previously suggested (Dieterich et al. 1997; Maki, 1997a), as the production of anti-tTG antibodies would continue as long as the tTG antigen were present (Sollid et al. 1997). In addition, as tTG is widely distributed and is released upon tissue damage, one would then expect that the immune response to tTG would be chronic and not regulated by gliadin. This concept could also explain why antibodies to tTG are more disease specific than those to gliadin (Sollid et al. 1997).

The detailed mechanism of the interaction between tTG and gliadin was later demonstrated. Using *in vitro* studies, it was found that tTG produced a selective deamidation of glutamine residues (from glutamine to negatively charged glutamate) of pepsin-trypsin digested gliadin (Molberg et al. 1998). The DQ2 alleles are known to preferentially bind to negatively charged amino acids. TTG-modified gliadin was found to increase DQ2 binding ten-fold by gut-derived T cells, but not peripheral T cells (Molberg et al. 1998). Interestingly, whereas non-enzymatic deamidated gliadin using acid/heat-treatment was non-toxic to coeliac mucosa (van de Kamer and Weijers, 1955), tTG deamidation produced epitopes which were advantageous for T cell recognition (Molberg et al. 1998). A plausible theory for the production of anti-tTG antibodies would be that tTG binds deamidated gliadin, forming complexes which are presented by DQ2-bearing cells to mucosal gliadin-specific T_H cells, which help tTG-specific B cells produce anti-tTG antibodies (Sollid et al. 1997). The association between HLA DQ2 and EmA, but not AGA, is further evidence for the underlying role of mucosal gliadin-specific T cells in the production of EmA (Maki et al. 1991; Maki, 1995).

Although it had already been demonstrated *in vitro* that CD mucosa, but not controls, produce EmA the mechanism was not known (Picarelli et al. 1996). The above findings indicate that the submucosa provides an ideal microenvironment for the

process of specific tTG-mediated gliadin modification, DQ2-binding and T cell recognition (Molberg et al. 1998). This theory provides a molecular explanation for the HLA DQ2 association in CD, but does not explain why not all DQ2 positive individuals develop the disease (van de Wal et al. 2000). It does not however demonstrate if deamidation would still occur if lysine residues were available. As previously described, tTG cross-links protein-bound glutamine and lysine residues and deamidation will only occur if lysine residues are unavailable (Folk, 1972). Under physiological conditions, it is probable that matrix proteins containing lysine residues would be available to prevent deamidation from occurring (Schuppan et al. 1998). It is also not known if physiological levels of tTG in normal or inflamed mucosa would be sufficient for deamidation to occur (Mowat, 1998). In addition, the finding of enhanced T cell recognition of tTG-gliadin in the mucosa, but not in the periphery, is curious as there has been no previous evidence that the repertoire of T cells are different in these locations (Mowat, 1998). An explanation for this finding might be that gut derived gliadin-specific T cells have a strong HLA-DQ2 restriction (Lundin et al. 1993; Maki, 1995), whereas peripheral gliadin-specific T cells do not have a similar HLA restriction bias (Gjertsen et al. 1994).

It is possible that the T cell response may first be directed to unmodified gluten, resulting in tissue damage and cytoplasmic tTG release in the submucosa generating more potent T cell stimulatory epitopes which would amplify the gluten-specific T cell response (van de Wal et al. 2000). Therefore, tTG, the target enzyme for the humoral response in CD patients, also profoundly influences cellular immune responses to gluten. The production of novel gliadin epitopes could play a role in breaking oral tolerance to both tTG and gluten.

Studies have implicated serum IgA anti-tTG antibodies in the pathogenesis of CD. It has been reported that these antibodies interfere with cellular differentiation and may

contribute directly to mucosal flattening (Halttunen and Maki, 1999). Other studies are now being conducted to investigate the role of tTG in the initiation of the disease. Although there are some unresolved issues with these evolving theories, enzymatic modification of proteins appears to be a new mechanism in the breaking of tolerance and in the pathogenesis of autoimmune diseases (Molberg et al. 1998).

2.4.4 The Transglutaminases in Inflammatory Bowel Disease

Both serum TG and tTG have been studied in the pathogenesis of IBD. An inverse correlation has been found between serum TG activity and the clinical severity as judged by the Crohn's disease activity index (D'Argenio et al. 1990; D'Argenio et al. 1995). Both types of TG were demonstrated in the extracellular matrix of inflamed tissue in Crohn's disease patients, and it was suggested that the fall in serum TG activity was due to its utilisation in the gut mucosa (D'Argenio et al. 1995). Later immunohistochemical studies in a rodent model demonstrated that both colonic mucosal tTG and serum TG activities were reduced in acute and chronic colitis, and that the level of reduction was related to the severity of the mucosal damage (D'Argenio et al. 1992). The mucosal tTG activity was predominantly in the repairing tissue and only minimal in damaged mucosa (D'Argenio et al. 1992). The reduction in mucosal TG was thought to reflect either an increased local utilisation for repairing processes, impaired enzyme synthesis or intra-luminal shedding of mucosal cells. It was suggested that the mechanism of histological recovery was that mucosal tTG migrated into the extracellular matrix and where it provided a protein network by cross-linking fibronectin, which was important for re-epithelisation and increased tensile strength of damaged tissue. These studies show the important role of both forms of TG in facilitating colonic mucosal repair and not in its destruction.

2.5 Diagnosis of Coeliac Disease

Accurate diagnosis of CD is important as it requires a life-long commitment to adhering to a GFD. Currently, the diagnosis of CD still requires the histological demonstration of an abnormal small intestinal mucosa. The anatomical lesion was first demonstrated using a peroral suction device at about the same time in England (Sakula and Shiner, 1957) and the United States of America (Crosby and Kugler, 1957). The recommended site to biopsy was in the jejunum, just distal to the duodenal-jejunal junction (Ligament of Treitz). Following the advent of upper GI endoscopy in the early 1970's, duodenal forceps biopsy largely replaced the suction technique as the preferred diagnostic tool (Shidrawi et al. 1994). Although small bowel mucosal biopsy is the "gold standard" by which the accuracy of serological tests is compared, villous atrophy is not entirely specific to CD. Other conditions with which it may be confused include cow's milk protein intolerance, post-infective enteritis, tropical sprue, Crohn's disease, intra-luminal bacterial overgrowth, and hypogammaglobulinaemia (Meeuwisse, 1970; Trier, 1998).

In 1969, the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) proposed a diagnostic protocol which required an initial characteristic biopsy while on a normal diet, histological improvement on gluten withdrawal and deterioration following gluten challenge (Meeuwisse, 1970). The protocol was simplified in 1989 (Walker-Smith et al. 1990), prompted by the availability of serological testing and evidence from the Italian Working Group for Paediatric Gastroenterology showing that subjects older than age 2 rarely required a gluten challenge to confirm the presence of CD (Guandalini et al. 1989). The new criteria required a characteristic initial biopsy followed by a definite, reasonably rapid clinical remission on a strict GFD with complete symptomatic relief (Walker-Smith et al. 1990). Biopsy findings were supported by the presence of 2/3 positive serological tests: AGA, ARA and/or EmA,

together with their disappearance in line with a clinical response. The finding of occasional false positive and negative results meant that the diagnosis could still not be established exclusively on the basis of positive serology. Gluten challenge was only deemed necessary under specified circumstances.

In adults, the symptomatic presentations of untreated CD vary widely and are not specific to the disease. Many subjects present during the third to fifth decades, but this may be delayed until later life (Trier, 1998). Efforts to standardise diagnosis have been confounded by the changing clinical pattern of the disease to milder forms (Ferguson et al. 1993; Logan et al. 1983), the finding that not all individuals with severe mucosal abnormalities respond to a strict GFD (Booth, 1974), and that gluten-sensitivity is not restricted to the presence of villous atrophy (Marsh, 1992).

The term “latent” CD was first introduced by Weinstein in 1974, to describe patients with DH and normal jejunal mucosa in whom typical coeliac histology could be induced following a 20-gram gluten challenge (Weinstein, 1974). The term is defined as patients with a normal small intestinal biopsy on a gluten-containing diet who in the past (or future) have had an abnormal small intestinal biopsy that improves following a GFD (Ferguson et al. 1993). Although a normal biopsy from a patient on a gluten-containing diet is generally thought to exclude CD, it has been shown that coeliac lesions may follow a normal biopsy (Collin et al. 1993; Maki et al. 1991; Maki et al. 1990). Patients with positive serology but normal duodenal histology are thought to represent “false-positives”, but in fact may represent latent CD. If these EmA positive patients are re-biopsied after an interval of about 2 years (or earlier if the patient develops symptoms), about 40-65% of them will have characteristic coeliac histology (Johnson et al. 1998; Collin et al. 1993; Cataldo et al. 1995). It was recommended that these individuals should not start a GFD until definite histological criteria are reached (Collin et al. 1993). Seropositive patients with “normal” biopsies using

conventional microscopy may have subtle abnormalities, such as raised IELs, detected using morphometric studies (Kaukinen et al. 1998; Holm et al. 1992). The term “silent” CD was proposed for patients who have characteristic intestinal villous changes, which return to normal on a GFD, in the absence of any clinical symptoms (Visakorpi, 1992).

The measurement of intestinal permeability has been a useful non-invasive tool in the assessment of subjects with symptoms of intestinal malabsorption. The technique was first described by Menzies *et al.*, and involved the differential urinary excretion of orally administered test sugars, a monosaccharide (rhamnose) and a disaccharide (lactulose) (Menzies et al. 1979). Neither of these sugars are hydrolysed by intestinal enzymes, and following their passive absorption, both are largely excreted unchanged in the urine. The absorption of rhamnose, was proportional to the villous surface area of the small bowel. A reduction in rhamnose absorption, with low urinary excretion, indicated the presence of villous atrophy (decreased absorptive area). The synthetic disaccharide, lactulose, was negligibly absorbed if the epithelium was intact, but paradoxically was increasingly absorbed in the presence of epithelial damage (raised urinary excretion), indicating leakiness of the abnormal mucosa to larger molecules. The ratio of the urinary excretion of lactulose/rhamnose (L/R) was significantly higher in untreated CD patients. The absorption of these sugars was therefore thought to reflect the integrity of the epithelial tight junctions. Sugar permeability tests have been found to be a sensitive tool in screening CD by some groups (Rostami et al. 1998; Vogelsang et al. 1995), but not by others (Catassi et al. 1997). The test lacks diagnostic specificity as raised results may also be found in dermatitis herpetiformis, Crohn’s disease, giardiasis, *Rotavirus* infection, tropical sprue and in non-steroidal anti-inflammatory drug-induced enteropathy (Bjarnason et al. 1995).

2.6 High Risk Groups

In developed countries, the prevalence of CD has been estimated to be between 1:200 and 1:400 (Mylotte et al. 1973; O'Farrelly, 2000). Certain auto-immune diseases such as IDDM, Addison's disease, Graves' disease and Sjogren's disease, which share the same HLA susceptibility genes (HLA B8 and DR3) with CD are at a higher risk of enteropathy (Collin et al. 1994) (**Table 2.1**). Screening of high risk groups appears to be justified by the findings that CD is associated with an increased frequency of lymphoma in the order of 40-100 fold (Holmes et al. 1989; Leonard et al. 1983) and also of small bowel adenocarcinoma, oesophageal and pharyngeal squamous carcinomas (Swinson et al. 1983). Malignancy may also develop in the presence of minimal mucosal pathology and a latent state (Marsh, 1997). Relatives of CD patients have been shown to have an approximately 10-fold increased risk of malignancy compared to age-matched population controls (Marsh, 1997). In addition, older or newly diagnosed CD patients presenting over the age of 50 years have a 1 in 10 chance of harbouring a lymphoma (Cooper et al. 1982).

Predictive characteristics for an enteropathy associated T cell lymphoma include male gender, a minimal or transient response to a GFD, and the absence of AGA (O'Farrelly et al. 1986). The diagnosis of a complicating lymphoma is often difficult and made late, because of a lack of serological markers and the presenting symptoms are similar to that of active CD. Early diagnosis of CD in at risk groups, who are often asymptomatic, would seem justified as adherence to a GFD for five years or more has a protective role in preventing malignancy at all sites associated with CD (Leonard et al. 1983). It is therefore important to recognise all CD patients even with minor symptoms and introduce a GFD.

Table 2.1: High Risk Groups to Screen for Coeliac Disease

High Risk Groups	Risk of CD
DH	30-100%
First-degree relatives of coeliac patients	10-20%
Neurological disease of unknown cause	16%
Selective IgA Deficiency	10%
Primary Biliary Cirrhosis	6%
IDDM	5.4%
Thyroid disease	5.4%
Epilepsy and Cerebral calcifications	5%
Down's Syndrome	3.9%
Sjogren's Syndrome	3.3%

2.6.1 Selective IgA Deficiency

Selective IgA deficiency (SIgAD) is the most common primary immunodeficiency, with a prevalence of 1:500 (0.2%) to 1:700 (0.14%) in the general population (Collin et al. 1992; Cataldo et al. 1999). In addition to a 10-fold higher risk of CD than the general population (Collin et al. 1992; Cataldo et al. 1999), it predisposes to recurrent infection and to autoimmune diseases (Cataldo et al. 1999; Ammann and Hong, 1970). It is uncertain why individuals with SIgAD are at a higher risk of CD, but it is possible that genetic factors and a lack of mucosal IgA reduces the exclusion of dietary antigens and lead to impaired immunological tolerance (Cataldo et al. 1999; Cataldo et al. 1998). In SIgAD, the clinical presentation, histology and response to GFD are identical to patients with normal serum IgA (Collin et al. 1992; Cataldo et al. 1999), however one study has

shown a 13% increased incidence of “silent” forms (Cataldo et al. 1998). SIgAD is known to produce false-negative IgA AGA and/or IgA EmA results (Cataldo et al. 1999; Beutner et al. 1989). IgG AGA, ARA and/or EmA have been found to be useful in diagnosing these patients and in monitoring their response to a GFD (Collin et al. 1992; Beutner et al. 1989; Sulkanen et al. 1998a). As about 6% of SIgAD patients may lack IgG AGA (Cataldo et al. 1998), small bowel biopsy should be performed whenever there is a high suspicion of CD in these patients (Cataldo et al. 1998). It has been recommended that total serum IgA concentrations should be measured when screening for CD to avoid the possibility of false-negative IgA serological results (Dickey et al. 1997; Cataldo et al. 1999; Cataldo et al. 1998).

2.6.2 Dermatitis Herpetiformis

Dermatitis herpetiformis is characterised by a symmetrical pruritic, blistering skin rash with granular subepidermal deposits of IgA in remote, uninvolved skin (Marks et al. 1966). The origin of these IgA deposits is unknown, however the strong association with CD has led to the hypothesis that this IgA is of gut origin (McCord and Hall, 1994). DH was first associated with CD by Marks *et al.* in 1966 (Marks et al. 1966). The presence of CD is difficult to suspect in DH patients as most are asymptomatic, and there is little laboratory evidence of malabsorption (Marks et al. 1966). These findings are thought to reflect the limited extent of the enteropathy in these patients (Volta et al. 1992; Lerner, 1991). The frequency of enteropathy in DH has been reported to be as low as 30% (Davies et al. 1978), but this has been disputed by Brow *et al.* who have shown that the enteropathy is always present if sufficient mucosal biopsies are taken (Brow et al. 1971). Indeed, DH patients with apparently normal intestinal biopsies can be induced to manifest characteristic biopsy appearances after a gluten challenge (Weinstein, 1974). The small intestinal histology in DH is indistinguishable from CD lesions (Marks et al. 1966), and both the skin and intestinal lesions respond to gluten withdrawal (Gawkrödger et al. 1984). DH patients on a normal diet have a 10-fold

increased risk of malignancy, predominantly lymphoma, compared with those on a GFD (Collin et al. 1996).

2.7 Spectrum of Serological Tests

Although small intestinal biopsy is still essential for the diagnosis of CD, it is invasive, time-consuming, expensive and may be unpleasant (Ascher et al. 1996). Less invasive screening tools, such as serology, are required for investigating patients with mild or atypical symptoms, for screening high risk groups, for timing biopsy following gluten challenge and for monitoring dietary compliance (Cataldo et al. 1995). The sensitivities and specificities of the various serological tests are shown in **Table 2.2**.

2.7.1 Anti-Gliadin Antibodies

Anti-gliadin antibodies were first described by Berger in 1958, and were the earliest discovered serologic markers associated with gluten sensitive enteropathy (Rossi and Tjota, 1998). AGA are directed against dietary gliadin which may be absorbed intact across the gut mucosa, as has been shown with ovalbumin, in healthy individuals (Husby et al. 1985). Serological techniques for measuring AGA have evolved and improved over the years. The ELISA technique, first introduced by Hekkens *et al.* in 1977, has become the most common technique because of its simplicity, low cost, objectivity and the possibility of analysing large batches of sera at a time (Rossi and Tjota, 1998). It also allows for sub-class identification of antibodies (Unsworth, 1996). Although gliadin is a complex mixture of proteins containing at least 40 components for a single variety of wheat (Rossi and Tjota, 1998), there is no evidence that the use of highly purified gliadin fractions as the coating antigen improves disease specificity (Unsworth et al. 1984). Our in-house AGA ELISA uses crude gliadin.

Untreated CD sera contain high levels of IgA, IgG and IgE AGA, but not usually IgM (Juto et al. 1985), unless the patient is IgA deficient (Savilahti et al. 1983). Raised concentrations of AGA are also found in intestinal secretions of CD patients and are predominantly of the IgA and IgM isotypes (Arranz et al. 1994). Serum AGA is thought to mostly originate from the gut mucosa on the basis of its molecular size (Troncone and Ferguson, 1991) and subclass distribution (Osman et al. 1996). In practice, only IgA and IgG AGA are used diagnostically but there has been controversy over the preferred sub-class (Lerner, 1991). The sensitivity of IgA AGA ranges from 46-100% and specificity from 86-100% (Unsworth et al. 1983; Bode and Gudmand-Hoyer, 1994; Volta et al. 1986; Volta et al. 1983). The sensitivity of IgG AGA ranges from 62-100% and specificity from 79-97% (Carroccio et al. 1996; Bode et al. 1993; Bode and Gudmand-Hoyer, 1994; Volta et al. 1983). The tests are often used in combination to take advantage of the high sensitivity of IgG and the high specificity of IgA AGA. When used in combination, the sensitivity increases to 77-100% and specificity to 80-99% (Unsworth et al. 1983; Scott et al. 1990; Bode et al. 1993; Bode and Gudmand-Hoyer, 1994). This approach also has the added benefit of detecting CD patients with SIgAD (Unsworth, 1996). IgA AGA may be normal in up to 16% of adult untreated CD patients (Scott et al. 1990), but few cases have both normal IgA and IgG AGA (Unsworth, 1996).

Both IgA and IgG AGA have a 100% sensitivity in CD patients under the age of 2 years (Savilahti et al. 1983) however, the sensitivities fall in older children to 52-64% and to 55-88% respectively (Savilahti et al. 1983; Lerner et al. 1994). AGA concentrations increase with age in healthy individuals, with levels rising from 12% in children to 35-40% in those aged 60-70 years (Lerner, 1991; Grodzinsky et al. 1992). Raised AGA concentrations should therefore be interpreted cautiously in older age groups. In addition, although gliadin is important in the pathogenesis of CD and a GFD is the treatment of choice, AGA are not specific to CD (Karpati et al. 1990). The presence of

AGA in healthy individuals or other disease states may be explained by the absorption of intact gliadin across the normal gut mucosa (Husby et al. 1985; Unsworth, 1996). Raised AGA levels in healthy individuals with normal intestinal biopsies may be transient (McMillan et al. 1996; Uibo et al. 1993), but it is also associated with a higher incidence of diarrhoea, chronic fatigue as well as a significantly lower serum folate than healthy controls (Arnason et al. 1992).

AGA are not HLA-associated (Troncone and Ferguson, 1991) and are not more common in atopic individuals (Hed et al. 1986). Raised IgG AGA concentrations have been found in skin disorders including atopic eczema, pemphigus, and pemphigoid (Troncone and Ferguson, 1991). Raised AGA has also been noted in Sjogren's syndrome, and in about 50% of patients with rheumatoid arthritis, raising the possibility that these individuals are more prone to NSAID-induced damage with increased small bowel mucosal permeability to gliadin (Troncone and Ferguson, 1991). Raised IgA AGA have also been found in Crohn's disease, cow's milk enteropathy and autoimmune enteropathy in association with an abnormal small bowel mucosa (Savilahti et al. 1983; Unsworth et al. 1983). IgA AGA is thought to be a better indicator of small bowel abnormalities than IgG AGA (Savilahti et al. 1983; Unsworth et al. 1983), but interestingly AGA is not elevated in intestinal T cell lymphoma (O'Farrelly et al. 1986).

2.7.2 Anti-Reticulin Antibodies

Antireticulin antibodies (ARA) were first described in adult CD and DH (Seah et al. 1971b) and in childhood CD patients (Seah and Fry, 1971a) by Seah *et al.* in 1971. ARA are detected using an immunofluorescence technique on a composite block of rat liver, kidney and stomach tissue - the same substrates used for autoantibody profile screening (Unsworth, 1996). Although five patterns of reticulin antibody fluorescence have been described, only the original pattern designated as R1-reticulin (R1-ARA) (Rizzetto and Doniach, 1973) is associated with CD (Eade et al. 1977). The different

staining patterns of ARA can be difficult to distinguish, and a definite positive R1-ARA result requires the pattern to be present in all three substrates (Unsworth, 1996).

The sensitivity of ARA has been disappointing in several studies following its initial optimism in diagnosing 85% of children with CD (Seah et al. 1973). Further studies in CD subjects have shown that the ARA sensitivity and specificity range from 44-100% and 92-100% respectively (Hallstrom, 1989; Volta et al. 1991; Kolho and Savilahti, 1997). In DH, the sensitivity is lower between 12-30% (Seah et al. 1973; Eterman and Feltkamp, 1978). Others have found that IgA R1-ARA positive samples are always EmA positive, but the converse is not always true (Unsworth, 1996). ARA appears to be highly specific to CD but only in experienced hands because of its difficult immunofluorescence interpretation. False positive IgA ARA have been reported in IDDM (Maki et al. 1984) and in Crohn's disease (Seah et al. 1973). The EmA test is now preferred as it is easier to read and more sensitive for CD without loss in specificity (Unsworth, 1996).

2.7.3 Anti-Endomysium Antibodies

Anti-endomysium antibodies, first described by Chorzelski *et al.* in 1983, are directed against the connective tissue surrounding smooth muscle fibres (Chorzelski et al. 1983). It has been proposed that the overlap in sensitivity and specificity between EmA and ARA suggest that they are in fact identical, but that the rodent tissue used for detecting ARA is not as sensitive as the primate substrate used for EmA (Hallstrom, 1989; Unsworth, 1996). Others have disagreed (Ferreira et al. 1992; Kolho and Savilahti, 1997). EmA are detected using an immunofluorescence technique with positive samples giving a honeycomb staining pattern in the connective tissue surrounding the myofibrils (Chorzelski et al. 1984; Unsworth, 1996). Interestingly, while ARA reacts with both human and rodent tissues (Maki et al. 1984), EmA are more species-specific and react only with the endomysium of the GI tract of primates

(Rossi et al. 1988). The EmA technique was initially developed using monkey oesophagus (MO) as the substrate (Chorzelski et al. 1984), but for ethical and cost reasons, there has been a need for alternative substrates.

In 1994, Ladinser *et al.* proposed the use of human umbilical cord (HUC) which is rich in matrix proteins surrounding the smooth muscle fibres in the wall of the umbilical vein and arteries (Ladinser et al. 1994). They reported that HUC had a 100% sensitivity and specificity compared to 90% with MO suggesting that human tissue was a more sensitive substrate than MO. Others have confirmed that the sensitivity of HUC is identical to (Rostami et al. 1999a; Kolho and Savilahti, 1997; Sacchetti et al. 1996; Volta et al. 1995), or superior to (Bottaro et al. 1997; Sategna-Guidetti et al. 1997) that of MO. Although other substrates have been proposed such as human oesophagus (Uibo et al. 1995) and human umbilical vein endothelial cells (Whelan et al. 1996), which are of comparable sensitivity and specificity to MO, they are not in common use. Our in-house method uses HUC, although kits containing frozen sections of MO or HUC are commercially available.

EmA antibodies are predominantly of the IgA class (Chorzelski et al. 1984). The IgG EmA test produces more false positives and should not be used (Unsworth, 1996), except in SIgAD where it has been shown to be sensitive (Sulkanen et al. 1998a). The EmA sensitivity and specificity range from 74-100% and 96-100% respectively (Hallstrom, 1989; Volta et al. 1991; Kolho and Savilahti, 1997; Valdimarsson et al. 1996; Cataldo et al. 1995). Not surprisingly, few studies have been able to reproduce the reported 100% EmA sensitivity and specificity (Kumar et al. 1989; Whelan et al. 1996; Dickey et al. 1997; Ferreira et al. 1992). The positive and negative predictive values (PPV and NPV) of EmA range from 79-100% and 95-100% respectively (Ferreira et al. 1992; Sategna-Guidetti et al. 1997; Valdimarsson et al. 1996).

EmA has not previously been positive in disease controls including IBD patients, but has been found in two asymptomatic patients with IDDM and Hashimoto's thyroiditis (Ferreira et al. 1992). Unfortunately both patients declined further investigation, but it is possible that they had silent CD which is more common in autoimmune diseases. False-positive EmA results have also been reported in a child with cow's milk enteropathy (Chan et al. 1994), in 6 symptomatic children (poor weight gain/growth, abdominal pain and vomiting) without histological abnormalities (Chan et al. 1994), and in two children with giardiasis (Rossi et al. 1988). Seroconversion and histological improvement followed anti-microbial treatment (Rossi et al. 1988).

Although the EmA test is highly sensitive, it fails to detect all gluten-sensitive individuals. False-negative results have been reported in untreated CD patients on a normal gluten-containing diet after the exclusion of SIgAD (Rostami et al. 1999a; Rostami et al. 1998). About 12% of CD children under the age of 2 years are falsely negative for IgA EmA (Burgin-Wolff et al. 1991; Beckett and Ciclitira, 1997). AGA is more sensitive in this age group (Ascher et al. 1996). EmA is clearly superior to AGA or ARA antibodies in selecting which patients should undergo definitive intestinal biopsy (Ferreira et al. 1992; McMillan et al. 1991).

2.7.4 Anti-Jejunal Antibodies

Serum IgA antibodies binding to human jejunum (JAB) were first described in DH patients by Karpati *et al.* in 1986, and subsequently in untreated CD patients (Karpati et al. 1986). They are detected using an immunofluorescent technique on cryostat sections of human foetal gut or normal human jejunum. The sensitivity in DH was 72% (Karpati et al. 1986) and in untreated CD was 93% (Karpati et al. 1990), with specificity of 100% (Karpati et al. 1986). Antibody titres did not correlate with the severity of the jejunal lesion. JAB disappeared after treatment with a GFD and reappeared in 90% of patients following gluten challenge (Karpati et al. 1990).

Like other tissue antibodies, the sensitivity of JAB falls in children under the age of 2 years to 71% (Karpati et al. 1990). Simultaneous determination of AGA, EmA, absorption studies, as well as ultrastructural studies, have suggested that JAB is identical to EmA but is different from AGA (Karpati et al. 1990; Karpati et al. 1992). It was also suggested that JAB might also be identical to ARA on the basis of staining characteristics (Karpati et al. 1986). Foetal jejunum has been proposed as an alternative substrate to monkey oesophagus because JAB are closely related to or identical to EmA (Kolho and Savilahti, 1997), but the use of foetal tissue would raise as many ethical issues as the use of MO. These antibodies are not in common use.

2.7.5 Anti-Calreticulin Antibodies

Karska *et al.* have shown that monoclonal AGA cross-react with epitopes on rat enterocytes which correspond to a distributed protein called calreticulin (Karska et al. 1995). Calreticulin is a multi-functional calcium-binding protein that can interact with integrin receptors and therefore has a potential role in extracellular matrix interaction. Calreticulin and gliadin share similar epitopes that could be recognised by anti-calreticulin (ACR) antibodies. Using an ELISA technique, a significant correlation between IgA ACR and AGA in CD patients has been demonstrated. Both ACR and AGA antibodies are significantly higher in untreated CD patients than treated CD patients or controls (Tuckova et al. 1997). It has been suggested that AGA may play a pathogenic role in CD by cross-reacting with enterocytes and that calreticulin may be one of the CD auto-antigens. These antibodies are not used in clinical practice.

2.7.6 Anti-Tissue transglutaminase Antibodies

Unlike EmA, anti-tTG antibodies can be measured using an objective, semi-quantitative ELISA method. The anti-tTG antibody ELISA uses guinea pig liver tTG, as currently a

human recombinant form of the enzyme is not yet commercially available. Ideally, the anti-tTG antibody ELISA should replicate EmA immunofluorescent findings. This was demonstrated in the first IgA anti-tTG antibody ELISA that had a 100% sensitivity and specificity in differentiating 25 EmA positive CD patients from 25 disease controls (Dieterich et al. 1997). Since then, the initial IgA anti-tTG antibody ELISA method (described in **section 3.1.2**) has been modified with some investigators attempting to activate tTG with calcium during the coating stage of the assay (Sulkanen et al. 1998b; Dieterich et al. 1998) while others have found this unnecessary (Lock et al. 1999a; Lock et al. 1999b; Troncone et al. 1999).

Reports of the sensitivity and specificity of the IgA anti-tTG antibody ELISA have ranged from 85-98% and 90-98% respectively (Sulkanen et al. 1998b; Dieterich et al. 1998; Lock et al. 1999b; Troncone et al. 1999; Biagi et al. 1999a). A PPV of 92% has been reported (Lock et al. 1999b). An *in vitro* radiobinding assay for measuring IgA anti-tTG antibodies (using ³⁵S-methionine-labelled TG) has also been found to be sensitive (97%) and specific (96%) (Bazzigaluppi et al. 1999). False positive IgA anti-tTG antibody results have been reported in disease controls with normal duodenal histology. These have included patients with abdominal pain and dyspepsia (n=4), gastroesophageal reflux (n=4), gastritis or gastric ulcer (n=4), and IBS (n=1) (Sulkanen et al. 1998b), unexplained anaemia (n=1), and a CD relative (n=1) (Lock et al. 1999b). No IBD or IDDM patients had raised IgA anti-tTG antibody concentrations (Sulkanen et al. 1998b). IgG anti-tTG antibodies appear to be reasonably specific to untreated CD (94-97%), but are not sensitive (21-42%) (Troncone et al. 1999; Bazzigaluppi et al. 1999).

Table 2.2: Sensitivities & Specificities of Serological Methods in Coeliac Disease

Serological Assay	Sensitivity	Specificity
IgA AGA	46-100%	86-100%
IgG AGA	55-100%	42-97%
IgA ARA	44-100%	92-100%
IgA JAB	93%	100%
IgA EmA	74-100%	96-100%
IgA anti-tTG	85-98%	90-98%
IgG anti-tTG	21-42%	94-97%

2.8 Serological Tests Following a Gluten Free Diet

Therapy with a GFD has been the mainstay in the treatment of CD for over 40 years. The clinical response to a GFD generally precedes mucosal recovery, which may be incomplete even after 2 years of dietary treatment (Grefte et al. 1988). Dietary non-compliance, whether intentional or inadvertent, is a common problem, and many of these patients are asymptomatic (Troncone et al. 1995). Monitoring compliance is difficult as objective markers, such as serology, do not consistently correlate with the underlying mucosal histology and dieticians are limited to a subjective assessment. Persistently positive IgA AGA and IgA EmA are useful indicators of continuing gluten intake, but the benefit of one over the other is still in dispute.

Serum IgA AGA concentrations decline following gluten withdrawal (usually within the first month) (Scott et al. 1990), eventually approaching that of controls (Troncone and Ferguson, 1991; Unsworth et al. 1983) but may persist in the intestine even in treated patients (Kelly et al. 1989). Raised IgG AGA levels may persist even with strict adherence to a GFD in both children (Savilahti et al. 1983; Unsworth et al. 1983) and adults (Kilander et al. 1987) despite documented healing of the enteropathy. Unlike the EmA, after prolonged gluten ingestion the number of AGA positive individuals falls despite continuing gluten ingestion and a flat mucosa (Burgin-Wolff et al. 1988). Therefore a normal AGA level in patients who have stopped their GFD for long periods, does not exclude persistent gluten intolerance.

The EmA is less practical for monitoring gluten withdrawal due to its subjective, qualitative reporting (negative or positive) unless multiple dilutions are performed to monitor the change in antibody titre. A decline in EmA titre following a GFD has been documented (Kumar et al. 1989), and usually starts within the first 4-12 months (Hallstrom, 1989). EmA results may not correspond to histological findings as in some patients the EmA may remain positive despite histological remission (Usselman and Loft, 1996; Volta et al. 1991) or are EmA negative in the presence of persistent jejunal lesions (Valentini et al. 1994). This indicates that the mucosa can recover in the presence of persisting antibodies, casting doubt about the role of antibodies in the pathogenesis of CD (Usselman and Loft, 1996). Others have found a good correlation between EmA result and histology following a GFD (Ladinser et al. 1994), and that the EmA corresponds more closely with histology than does the AGA (Lerner et al. 1994).

Re-appearance of the EmA is a reliable marker of mucosal relapse following gluten challenge, and it may also precede the appearance of villous atrophy (Burgin-Wolff et al. 1991). EmA positivity may precede (Cataldo et al. 1995) or follow a rise in AGA concentration (Troncone et al. 1995; Burgin-Wolff et al. 1988). In general, the IgA

AGA appears to be better than the EmA at monitoring the initial response to gluten withdrawal as it may become negative earlier than EmA, but the EmA is more sensitive at detecting mucosal relapse (often silent) after periods of gluten ingestion (Cataldo et al. 1995). IgA ARA titres fall rapidly following a GFD (usually within the first month), and re-appear on a gluten challenge (Hallstrom, 1989). The change in IgA anti-tTG antibody concentrations after dietary therapy has not yet been widely studied, but a significant fall in antibody concentration has been reported in treated CD (Dieterich et al. 1997; Dieterich et al. 1998). In the next chapter, stages in the development of the IgA and IgG anti-tTG ELISAs will be discussed.

CHAPTER 3: DEVELOPMENT OF THE IgA & IgG ANTI-TISSUE TRANSGLUTAMINASE ANTIBODY ELISAS

3.1 IgA Anti-tTG Antibody ELISA Method Development

3.1.1 Methodology Background

At the start of this research project, there was no existing in-house ELISA method for measuring IgA anti-tTG antibody concentrations. The previously published method by Dieterich *et al.* (Dieterich et al. 1997) was used as a baseline to begin evaluating the ELISA in our population of CD patients and controls. In brief, this method involved coating 96-well microtiter plates (Nunc, Wiesbaden, Germany) with 10 µg/ml guinea pig liver tTG (Sigma) diluted in a phosphate buffered saline (PBS) coating buffer. Incubation was for 2 hours at 37°C. Unreacted sites were blocked overnight with PBS containing 1% bovine serum albumin (BSA) at 4°C. Patient and control sera were diluted in a diluent of PBS/0.05% Tween 20 and incubated for 1 hour at room temperature (RT). Plates were washed for 3 cycles and then a peroxidase-conjugated antibody to human IgA (Dianova) diluted at 1/1000 in diluent solution was added. Incubation was for 1 hour at RT. Unbound antibodies were removed by another 3 wash cycle, and colour development was by the addition of 200 µl 0.1 M sodium citrate, 1 mg/ml o-phenylenediamine-hydrochloride, 0.06% H₂O₂, pH 4.2, for 30 minutes at RT. The absorbance was read on an ELISA reader (MRX, Dynatech laboratories, Germany) at 450 nm.

Dr. Kenneth Humphreys had already initiated the development of an in-house ELISA. Further method development and validation were performed with technical assistance

from and collaboration with him and Dr. Marian Aldhous. Each stage of the development was optimised with consideration given to plate type, concentration of coating antigen, time and temperature of coating, blocking conditions, sample dilution, conjugate concentration, incubation times, and stability and storage conditions. Only the upper reference limit was diagnostically significant and this was calculated from a cohort of anonymous blood donors. The inter- and intra-assay coefficients of variation (CVs) of the assay were determined and the performance of the assay was compared to that of a commercially available IgA anti-tTG antibody kit.

3.1.2 General Principle of ELISA Methods

Non-competitive ELISAs are used to measure antigen-specific or total IgA, or IgG in serum and/or gut secretions. The solid phase is formed by the plastic surface of 96-well ELISA plates, with different plastics having different binding characteristics. The plates are coated with a specific antigen and are incubated so that the antigen could bind to the plastic. After washing off excess unbound antigen, the plates are incubated with a protein-containing solution to “block” any non-specific binding sites. The blocking solution is then decanted and test samples for the measurement of class specific anti-human Ig or food antibodies are added and bind to the antigen on the solid phase. Samples for total Ig quantitation are added to the plates in doubling dilutions in order to cover the wide range of concentrations found, while those samples for antigen specific analysis are added at a single pre-determined dilution. All samples are analysed against a reference standard, with a designated antibody concentration, which is added in duplicate, in doubling dilutions, to the first two columns of each plate. Blank wells, consisting of diluent solution, and quality control samples are included on each plate. All samples and solutions are added in a volume of 100 µl/well unless otherwise stated.

The plates are incubated during which time binding take place between the coating antigen and the specific antibody present in the serum or WGLF. The plates are then

washed with a mild detergent solution to disrupt non-specific interactions. A class specific anti-human antibody conjugated with alkaline phosphatase is then added to each plate, at a pre-determined dilution, incubated and washed again. The addition of a chromogenic substrate for alkaline phosphatase, p-nitrophenyl phosphate in a diethanolamine buffer (DEA), to the plates results in the enzymatic conversion from a colourless to a yellow coloured substrate. The optical density (OD) of the substrate is measured spectrophotometrically on an ELISA reader using a test filter of 405 nm with a reference filter of 630 nm. The plates are read using assay specific programs on Microsoft Revelations software, when the top reference standard reaches an OD of 1.00. The program subtracts the OD reading of the blank samples from each well. Test sample concentrations are calculated automatically by the program based on the reference standard curve.

3.1.3 Optimal Coating Conditions

The coating antigen used was tTG from guinea pig liver (Sigma, T5398) which shared 80% sequence homology with the human enzyme (Ichinose et al. 1990). Unfortunately a human recombinant form of the enzyme was not yet commercially available. Without a purified standard of human antiserum to tTG with a known concentration of specific IgA anti-tTG, it was not possible to measure absolute antibody concentrations by ELISA. The tTG antigen was available in dry powder form in bottles of 0.5, 1.0 and 2.0 units. As the contents of a 2.0 unit bottle weighed only about 1 mg, it was impractical to weigh out small quantities of powder, and therefore the entire contents was diluted in one batch. The protein content (in mg) of each bottle was calculated from the units/mg protein data supplied. The optimal coating solution and concentration of tTG were investigated.

In the GI laboratory, in-house ELISAs for antibodies to specific antigens typically used Dynatech Immulon 2 high protein binding plates with coating antigen dissolved in an alkaline carbonate-bicarbonate (CB) coating buffer (pH 9.6). This differed from the



PBS coating buffer (pH 7.4) used in the Dieterich method. To investigate the optimal coating buffer solution, 1 plate was coated with 10 µg/ml of tTG in CB coating buffer and the other using PBS coating buffer. Both plates were blocked with 1% haemoglobin (Hb) in diluent (0.9% NaCl, 0.05% Tween 20). Three serum samples (96/0693, 96/0851, 96/0850) were doubly diluted from 1/25 (in duplicate) in diluent and 100 µl/well were added to each plate. Incubation was for 3 hours at 37°C. After colour development, the results showed that the OD responses were higher in all 3 samples when plates were coated with CB compared to PBS coating buffer (**Figure 3.1**). A CB coating buffer was therefore used, in preference to PBS, to reconstitute the tTG.

To determine if the antigen could be reconstituted in CB coating buffer and then stored, a small volume of the stock solution was briefly stored at -20°C and then thawed. A stock solution of 1 mg/ml of tTG in CB coating buffer was prepared. One half of an Immulon 2 plate was coated with the stored pre-diluted coating antigen and the other with freshly diluted coating antigen. Following an overnight incubation at 4°C in a covered moist box, the plate was washed for 3 cycles with the multi-reagent washer. Test samples, diluted at 1/25 in diluent solution, were added (100 µl/well) in doubling dilutions to corresponding wells on each half of the plate. After another overnight incubation at 4°C and wash cycle, substrate was added and the plates were read on the ELISA reader. The results showed that the OD readings of test samples measured on plates coated with pre-diluted antigen were lower than those coated with freshly prepared antigen. The coating antigen was therefore prepared fresh at all times and batches of plates were made.

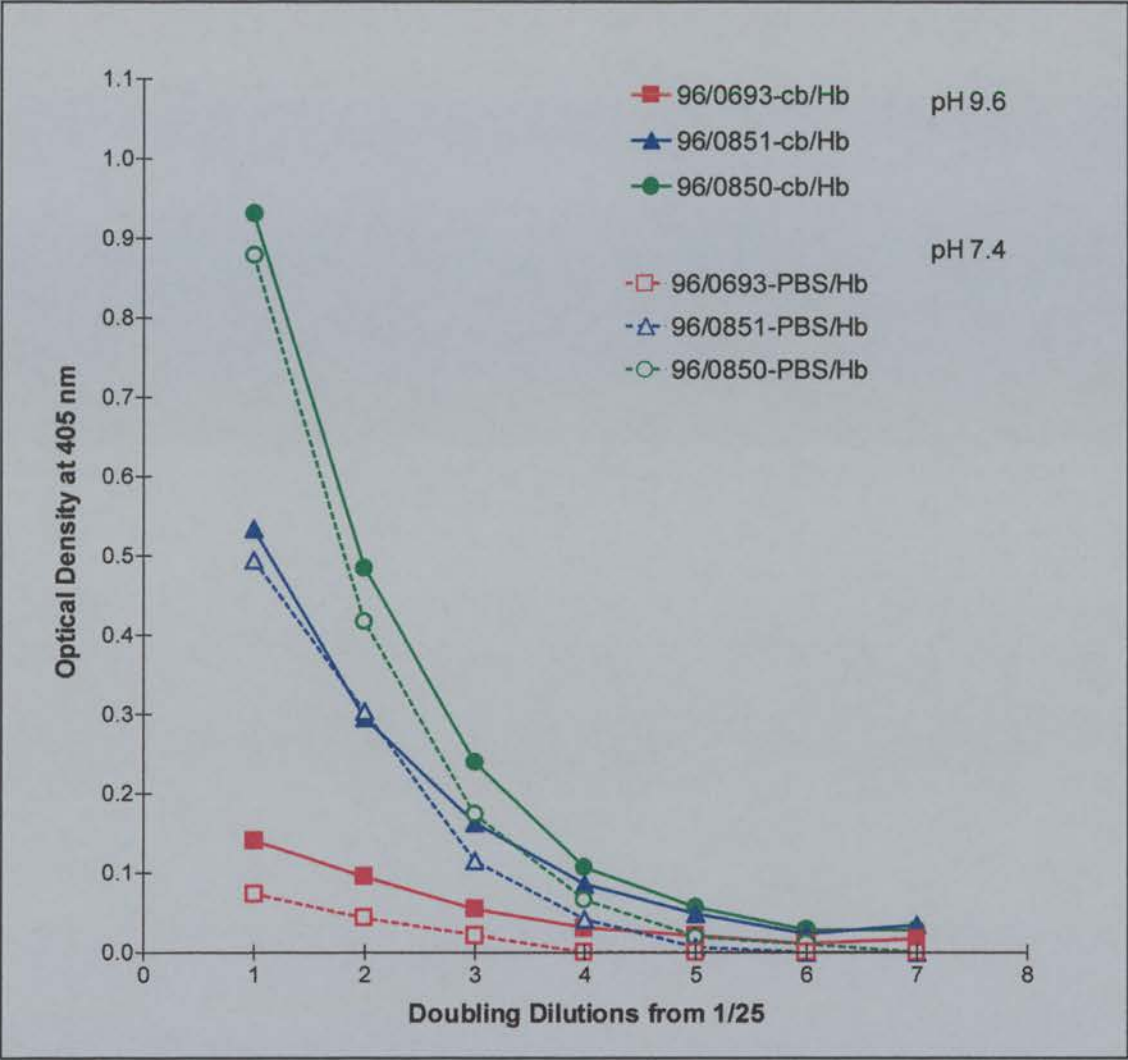


Figure 3.1 Comparison of CB & PBS coating buffers using 3 serum samples doubly diluted from 1/25. The OD responses were higher in all 3 samples when plates were coated with CB compared to PBS coating. A CB coating buffer was therefore used, in preference to PBS, to reconstitute the tTG.

The optimal coating concentration of tTG was determined by coating 2 Immulon 2 plates with doubling dilutions of tTG (made from the stock solution) from 20 µg/ml to 0.02 µg/ml in duplicate columns. The plates were incubated and then blocked with 1% BSA. The test serum sample, from an untreated CD patient with a positive IgA EmA, was added to the plates in doubling dilutions from 1/25 to each column. An optimal coating concentration of 10 µg/ml was found as above this coating concentration, no further increase in OD response was observed and a rapid decline in OD readings was noted below this concentration (**Figure 3.2a**). In addition, the coating concentration of 10 µg/ml produced the best dilution curve (**Figure 3.2b**). A 2.0 unit bottle of tTG, diluted at 10 µg/ml, coated 9 plates. Storage of some of these plates allowed us to observe changes in the plate performance with time.

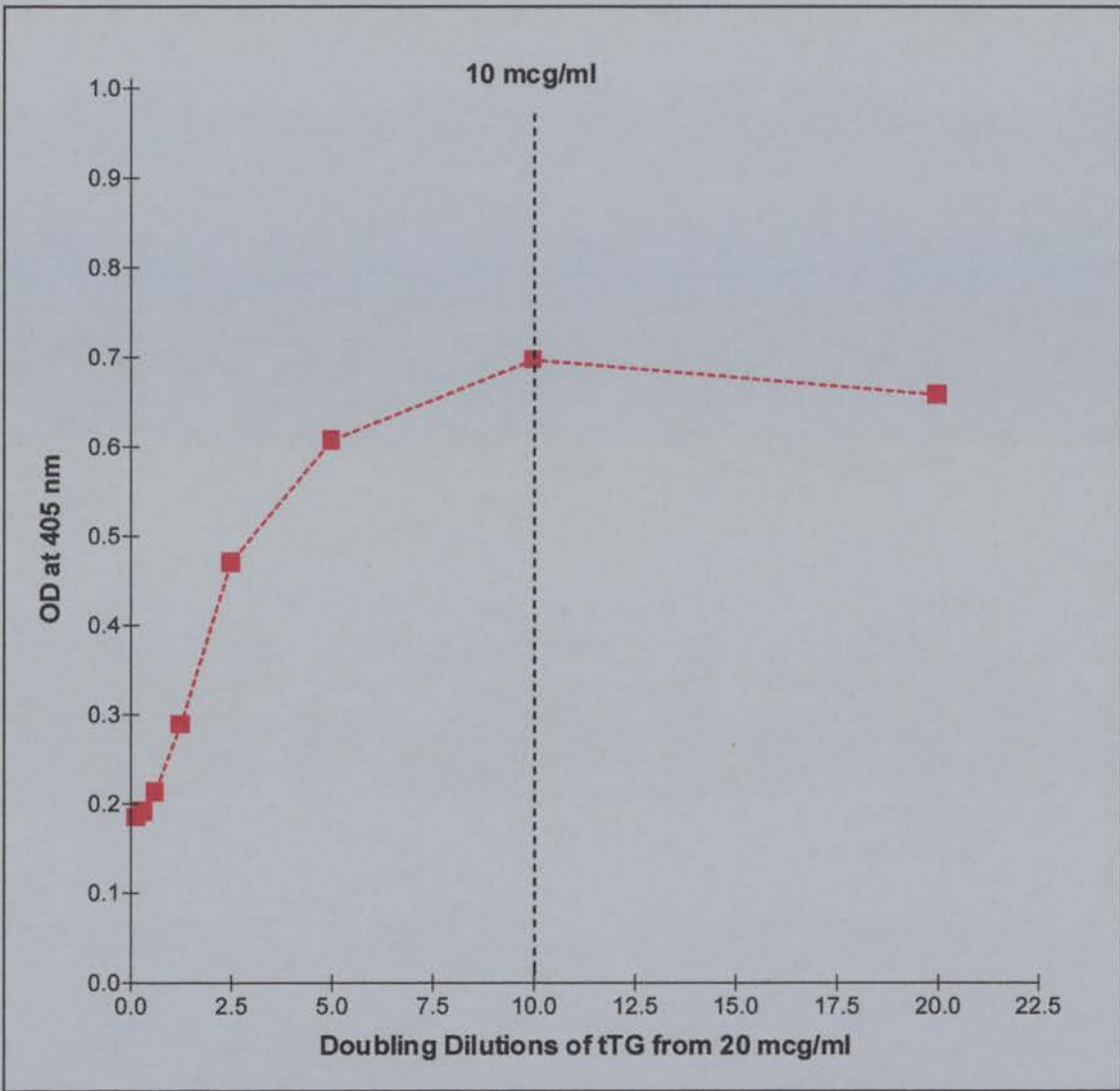


Figure 3.2a: Determination of the optimal tTG coating concentration. The graph shows the OD readings of a test serum sample from an IgA EmA positive untreated CD patient using plates coated with doubling dilutions of tTG from 20 $\mu\text{g/ml}$ to 0.02 $\mu\text{g/ml}$. An optimal coating concentration of 10 $\mu\text{g/ml}$ was found as above this coating concentration, no further increase in OD response was observed and a rapid decline in OD readings was noted below this concentration.

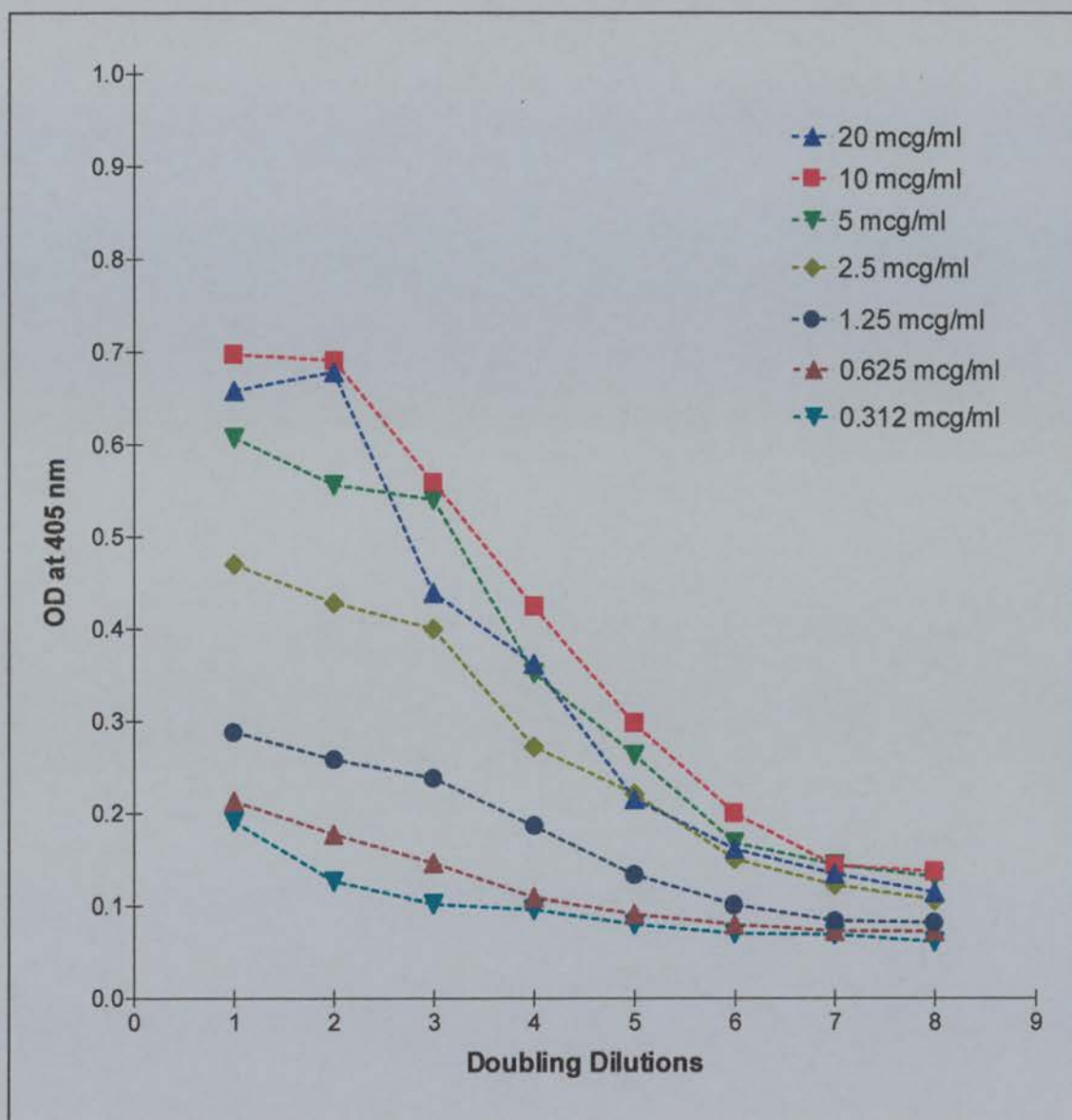


Figure 3.2b: Dilution curves of different concentrations of tTG. The OD readings of an IgA EmA positive sample was studied using plates coated with doubling dilutions of tTG from 20 $\mu\text{g/ml}$ to 0.312 $\mu\text{g/ml}$. The graph demonstrates that the optimal coating concentration of 10 $\mu\text{g/ml}$ also produced the best dilution curve.

3.1.4 Optimal Blocking Conditions

In ELISA methods, the plates are blocked to avoid non-specific reactions between the antibodies in the test sample and the plate. Most blocking solutions contain a relatively inert protein, which should not interact with antibodies in the test sample. The two blank wells in the top left-hand corner of an ELISA plate are filled with diluent solution only, and therefore the OD readings of these wells give an indication of the background of the assay. The OD readings of the blank values give the best indication of how well a plate is blocked (Dr. Marian Aldhous personal communication). The average blank value is subtracted from all of the OD readings on the plate and therefore a low blank value is desirable.

The 3 OD readings of different blocking solutions (1% BSA, 1% Hb and 1% gelatin) were compared. The average blank values for these blocking solutions were 0.058, 0.064 and 0.061 respectively. The 1% BSA blocking consistently had the lowest blank values on several different plates. There was some concern however, about using 1% BSA as the blocking solution as in theory, both healthy and CD subjects may have antibodies to BSA from encounter with the dietary antigens. Indeed, CD patients are known to have circulating antibodies to a number of food antibodies. Although human albumin may have been a better blocking agent, cost prohibited its routine use, and therefore 1% BSA was used as the blocking agent.

In a preliminary study to investigate the presence of serum IgA antibodies to BSA, plates were coated with 1% BSA in PBS and other plates with 10 µg/ml of tTG in PBS, both with a 1% Hb block. The same 3 serum samples were studied. Anti-human IgA conjugate, diluted at 1/800, was added (100µl/well). Substrate solution was added, and plates were developed when the top dilution reached an OD of 1.00. The results showed that the time for development of the plate coated with 1% BSA in PBS was much longer (61 minutes) compared with 5 minutes for the plate coated with tTG in PBS (**Figure**

3.3a). This suggests that although some individuals may have IgA serum antibodies to BSA, these do not contribute significantly when tTG-coated plates are blocked with BSA as OD responses to BSA are much slower than those to tTG.

The effect of BSA in the blocking solution on OD responses was also studied when BSA was substituted with Hb and when it was omitted. Using the same 3 serum samples, plates coated with 10 µg/ml of tTG in CB buffer were blocked with either 1% BSA, 1% Hb or no block. The ODs of all 3 samples were highest when plates were blocked with Hb compared to those blocked with BSA or no blocking solution (**Figure 3.3b**).

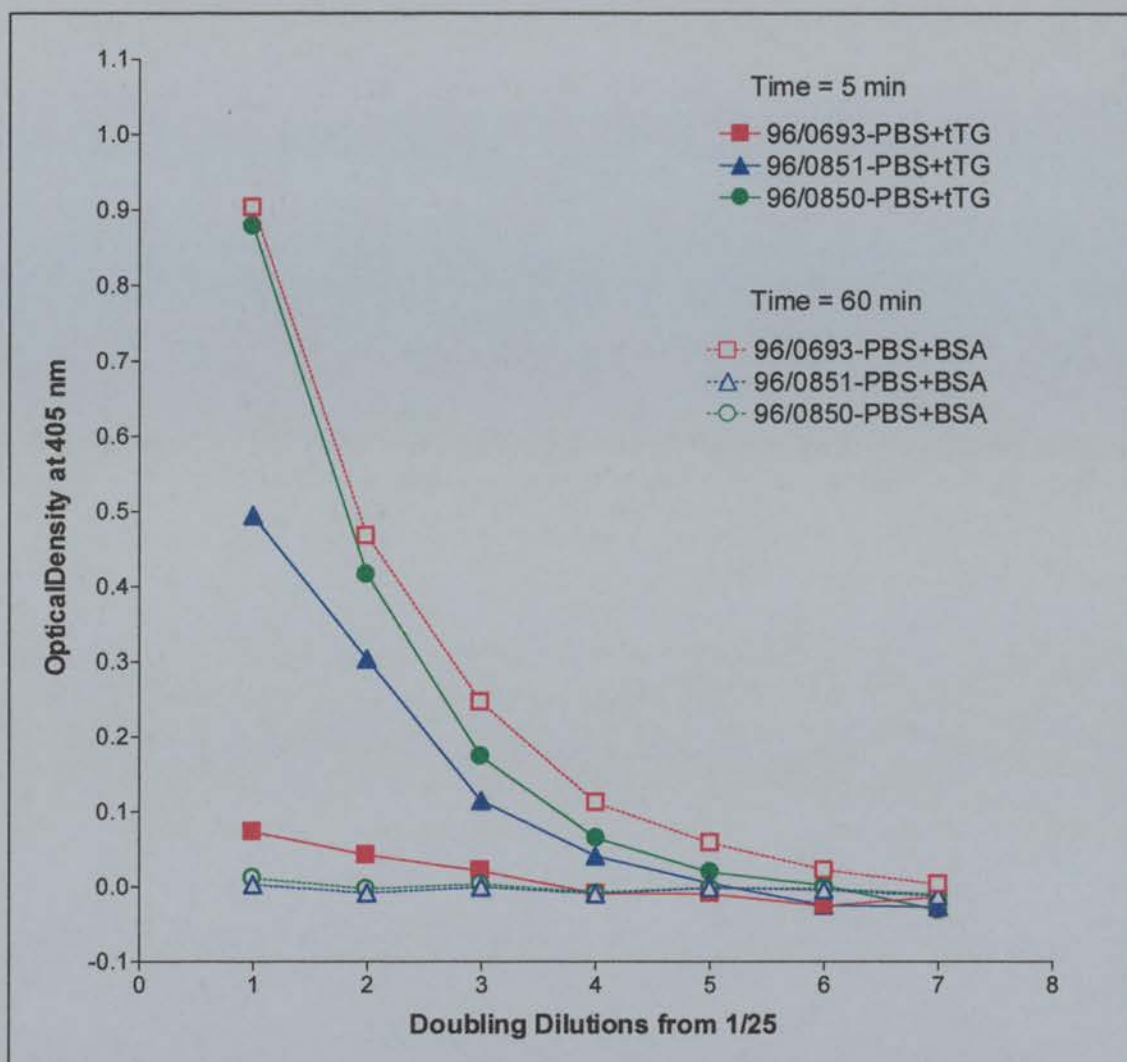


Figure 3.3a: Late detection of anti-BSA antibodies in the presence of tTG. The graph demonstrates the OD responses of 3 serum samples at doubling dilutions from 1/25 using plates coated with 10 μ g/ml of tTG in PBS and other plates with 1% BSA in PBS. The plate coated with 1% BSA in PBS took much longer to develop (61 minutes) than the plate coated with tTG in PBS (5minutes). This suggests that IgA anti-BSA antibodies do not contribute significantly when tTG-coated plates are blocked with BSA as OD responses to BSA are much slower than those to tTG.

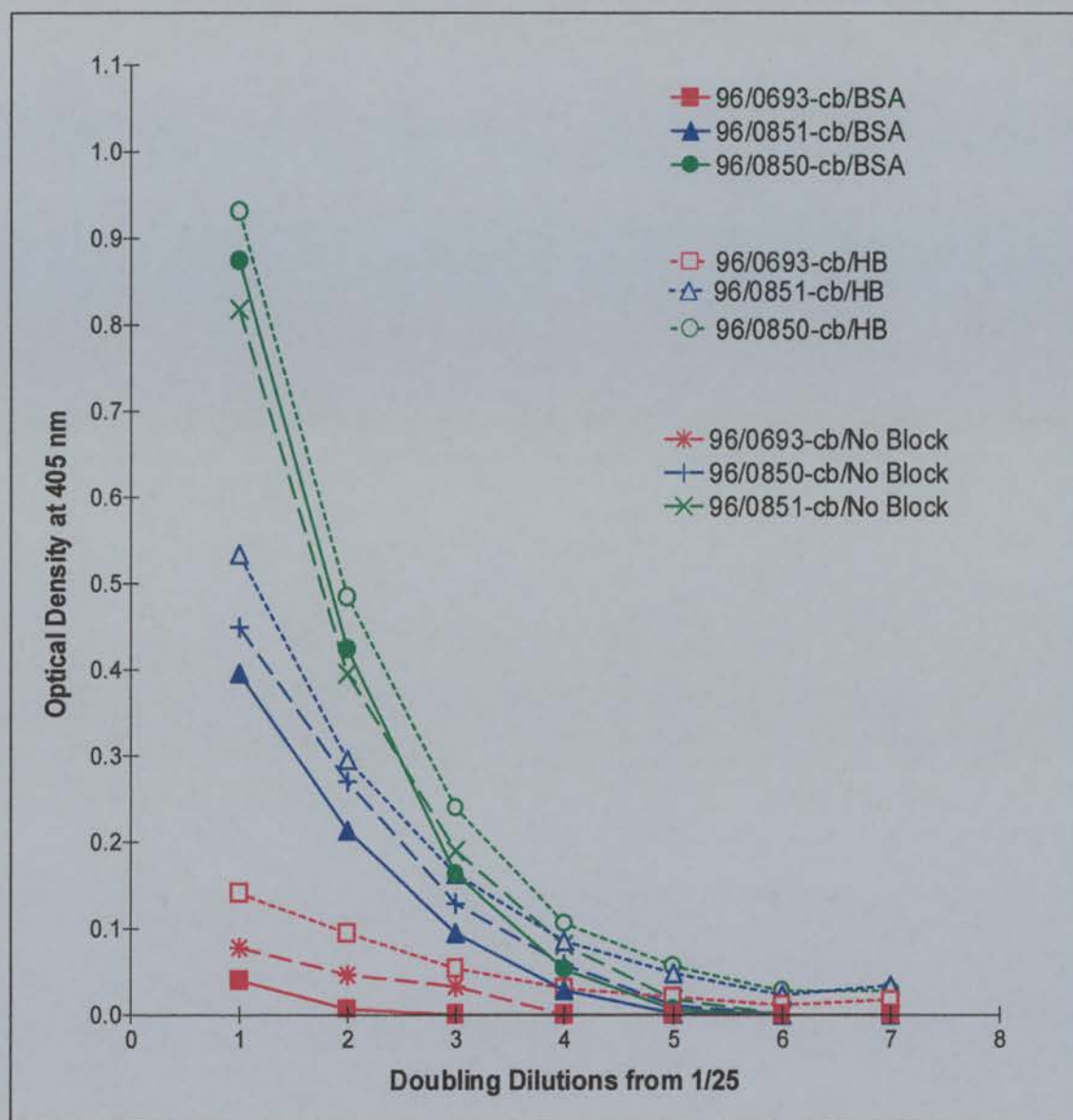


Figure 3.3b: Comparison of blocking solutions. The graph shows the effect on OD responses to 3 serum samples when similarly coated plates are blocked with different solutions: BSA, Hb or no block. The OD readings of all 3 samples were highest when plates were blocked with Hb compared to those blocked with BSA or no blocking solution.

During the latter stages of this project, having accumulated sufficient numbers of untreated CD patients to analyse, the issue of the presence of circulating BSA antibodies in the CD patients and controls was revisited. Plates were prepared as follows:

- Plate 1: Coating: CB (pH 9.6), 10 µg/ml tTG; Block: 1 % BSA block
- Plate 2: Coating: CB (pH 9.6), no tTG; Block: 1 % BSA block
- Plate 3: Coating: CB (pH 9.6), no tTG; Block: PBS

Sera from biopsy-proven, untreated CD patients (n=20) and patients with normal duodenal biopsies (n=20) were diluted at 1/100 and 1/50 respectively. The IgA conjugate was added at a dilution of 1/800 to all plates. Substrate was added to plate 1 first and the time taken for colour development was recorded. Substrate was then added to plates 2 and 3, and colour development was performed at the same length of time as that required for plate 1. The results showed that both CD and normal individuals may have IgA anti-BSA antibodies, but that only a small proportion of the OD in the duration of time required for the IgA anti-tTG assay was due to a reaction with BSA in the blocking solution (**Figure 3.4a & 3.4b**).

To investigate if the reaction between serum IgA and BSA in the blocking solution could be reduced, 2 plates were coated with 10 µg/ml of tTG in CB buffer and blocked with 1% BSA. For one plate, the blocking solution was decanted and the plate was blotted dry while the other plate was washed for 3 cycles. Serum samples were added at 1/50 and 1/100 to both plates, incubated and IgA conjugate was added at 1/800. Following addition of the substrate, the top standard reached an OD of 1.00 within 35 seconds in the washed plate compared to 8 minutes 42 seconds for the unwashed plate. As the plates developed too rapidly after removing the BSA by washing, it was thought to be unwise to do this before adding the test serum samples for analysis.

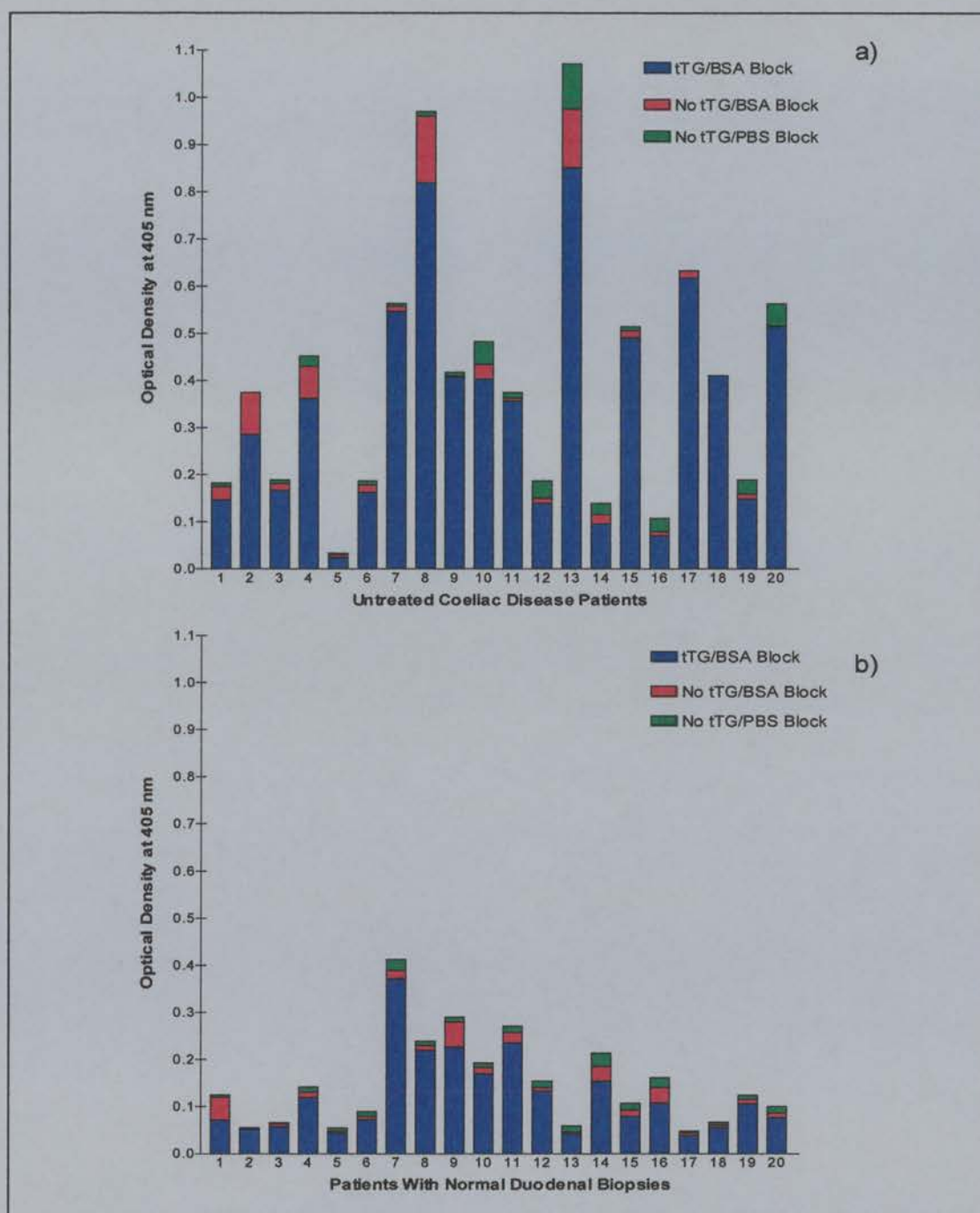


Figure 3.4a: Presence of anti-BSA antibodies in untreated coeliac disease patients

Figure 3.4b: Presence of anti-BSA antibodies in non-coeliac disease patients

3.1.5 Selection of a Reference Standard

In the absence of a purified standard with a known antibody concentration, a sample with high OD readings reflecting a high IgA anti-tTG antibody concentration was used. This standard was designated an arbitrary concentration in units/ml and doubling dilutions of this sample produced a reference standard curve. The OD readings of test samples were compared to the standard curve to obtain the antibody concentration. For this reason, the ELISA was semi-quantitative.

To select a sample with suitably high OD readings as the reference standard, stored sera from untreated CD patients were tested for OD response to tTG. Included in these sera was the currently used standard in the IgA AGA assay that showed very high OD responses to tTG. This CD patient, on a normal gluten-containing diet, had already given written consent for hepatitis and HIV testing. Confirmation of consent and negative infection screen were available in the QC documentation protocol file. In addition, there appeared to be a sufficient volume of this sample to be used for both the anti-tTG and AGA assays. This serum sample had been stored in 2 ml aliquots at -20°C. It was further divided into 50 µl aliquots in small Eppendorf tubes and stored at -20°C. Since antibodies are known to be most stable when specimens are frozen and used only once, aliquots were discarded after use. The same serum standard was used in the WGLF IgA anti-tTG ELISA due to the large volume of WGLF that would have been required for this purpose. Fortunately, there was enough standard serum available to allow completion of all of the test sample analysis with a single reference material.

3.1.6 Upper Reference Limits of IgA Anti-tTG in Serum & WGLF

Plasma samples from 106 anonymous blood donors were obtained with the co-operation of the haematologists. These samples were all labelled numerically and stored at -20°C until analysis. As it was not known if IgA anti-tTG antibody concentrations differed in serum and plasma, antibody levels in 18 paired samples (stored at -20°C) were compared. All samples were diluted at 1/50 and 1/100. The results showed that there were no discrepancies between antibody concentrations in serum and plasma. In addition, there was a significant positive correlation between concentrations between paired serum and plasma samples ($r^2=0.82$, $p<0.0001$) (**Figure 3.5a**). The donor plasma samples were therefore used to reflect IgA anti-tTG concentrations in serum. Others have also used randomly selected blood donor samples (without small intestinal biopsies) to obtain their reference range (Lock et al. 1999b).

The IgA anti-tTG antibody concentrations were measured in all donor plasma samples at dilutions of 1/50 and 1/100. The frequency distribution of the antibody concentrations is shown in **Figure 3.5b**. The majority of donors (84%) had very low IgA anti-tTG antibody concentrations less than 1000 units/ml. Only 4 (4%) subjects had an IgA anti-tTG antibody concentration greater than 4000 units/ml. One donor had a very high IgA anti-tTG antibody concentration of 31700 units/ml and subsequent testing found a positive IgA EmA result. Unfortunately, as the donor testing was done anonymously, there is no way to confirm if this individual had untreated CD. Others have also reported a high prevalence of CD in apparently healthy blood donors (Rostami et al. 1999). The histogram showed that this data was not normally distributed, and therefore a non-parametric method (Jones and Payne, 1997b) was used to calculate the upper reference limit. Using the 95th percentile, an upper reference limit in serum of 2950 units/ml was obtained.

The upper reference limit of IgA anti-tTG antibody concentrations in WGLF was determined later using the same non-parametric method. For this analysis, WGLF from 36 patients with irritable bowel syndrome and 13 healthy volunteers were analysed. An upper reference range of 110 units/ml was obtained at the 95th percentile.

3.1.7 Selection of Quality Control Sera

Quality control serum samples were used on each plate to ensure that the reference standard was performing reproducibly. To obtain suitable samples, sera from untreated CD and non-CD patients were tested at 1/50 and 1/100. The plates were read when the top standard reached an OD of 1.00. Samples showing appropriate OD responses to tTG were selected for use as a HiQC (above the reference range) and LoQC (within the reference range). Like the reference standard, QC samples were aliquotted, frozen at -20°C and used only once. The performance of the HiQC and LoQC were monitored by recording and graphing the QC results after each ELISA run to determine the within and batch-to-batch variation. For the same reasons discussed in **section 3.1.5**, the same serum QC samples were used in the WGLF IgA anti-tTG ELISA.

The HiQC sample used in the development of the IgA anti-tTG assay was the same sample used as the LoQC sample in the IgA AGA assay. Unlike the standard sample, this sample was in limited supply and this had not been appreciated at the time it was selected. This meant that the HiQC sample had to be replaced in the latter stages of the study. To ensure that assays performed with the original HiQC would be comparable in performance to later assays, the original HiQC was overlapped with a newly selected HiQC before supplies were finished. I did note, however, for future development of the IgG anti-tTG assay that it was not advisable to use either standard or QC material in common with other routinely performed assays.

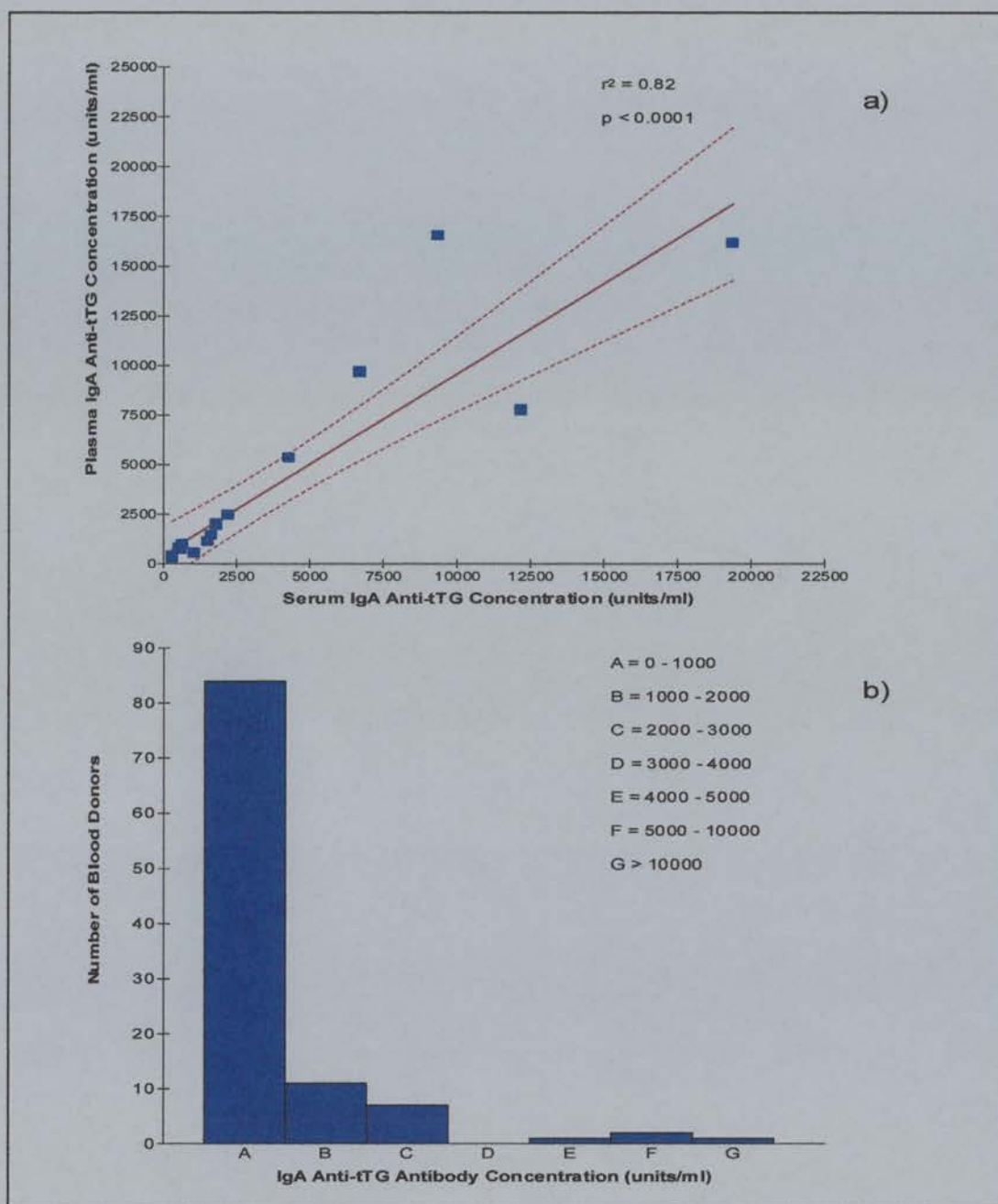


Figure 3.5a: Comparison of IgA anti-tTG antibody concentrations in serum & plasma

Figure 3.5b: Frequency distribution of IgA anti-tTG antibody concentrations in blood donors

3.1.8 Optimal Sample Dilution

Test serum samples were stored either at -20°C or -70°C and required about 20 minutes to thaw to RT. An optimal sample dilution should give at least 2 ODs parallel to the standard curve. To determine the optimal sample dilution, test sera were doubly diluted in polypropylene tubes initially from 1/25 to 1/200. For most samples, the OD readings at a 1/200 dilution were too low for comparison with the reference standard. The OD readings at dilutions of 1/50 and 1/100 for most samples were parallel to the standard curve. In cases where the OD readings of test serum samples were above the top standard, further doubling dilutions were performed until OD readings were in the range of the reference standard.

Test WGLF samples were initially doubly diluted from neat to 1/64 dilution. WGLF samples from CD patients had widely varying ODs and all required doubling dilutions from 1/2 to 1/64 to ensure that at least 2 dilutions were parallel to the serum standard curve. For non-CD WGLF samples, dilutions of 1/2 and 1/4 were usually sufficient to obtain ODs within the range of the serum standard.

3.1.9 Optimal Conjugate Dilution

The optimum concentration of the IgA alkaline phosphatase conjugate was determined by adding doubling dilutions (from 1/200) of a positive serum sample in rows down a tTG-coated plate. A blank sample (diluent solution) was added to the top row of the plate. The conjugate was added to duplicate columns down the plate at concentrations of 1/400, 1/800 and 1/1000. The optimum conjugate dilution of 1/800 was selected, as above this dilution there was a sharp decline in OD readings noted.

3.1.10 Stability & Storage Conditions

In another series of experiments, 7 serum samples were analysed, of which 2 samples were from IgA EmA positive untreated CD patients. The optimal coating temperature and storage conditions were investigated using CB coating system at 4°C and 37°C. As tTG could not be stored after re-constitution, it was more convenient to coat several plates each time in batches. Like many other serological tests, it was also more cost effective and efficient to perform the assay in batches. The stability of the CB coated/BSA blocked plates was studied after freezing.

The 7 samples were diluted at 1/50 in tTG diluent and the OD responses were compared. This showed that the OD responses were higher following coating at 37°C for 4/5 of the EmA negative samples, but were higher following coating at 4°C for the 2 EmA positive samples. There was no reduction in OD responses after freezing at -20°C for 24 hours (**Figure 3.6a**). The stability of plates stored at -20°C was studied for longer periods of time by plotting the OD responses of the HiQC used on each plate for a batch stored for up to 4 months. This showed that plates appeared to be stable after storage at this temperature for up to 3 weeks, but following this there was a decline in OD response (**Figure 3.6b**). In the initial stages of the project, batches of plates were prepared every 3 to 4 week, however in the latter stages when the number of test samples to be analysed reduced, plates were coated weekly.

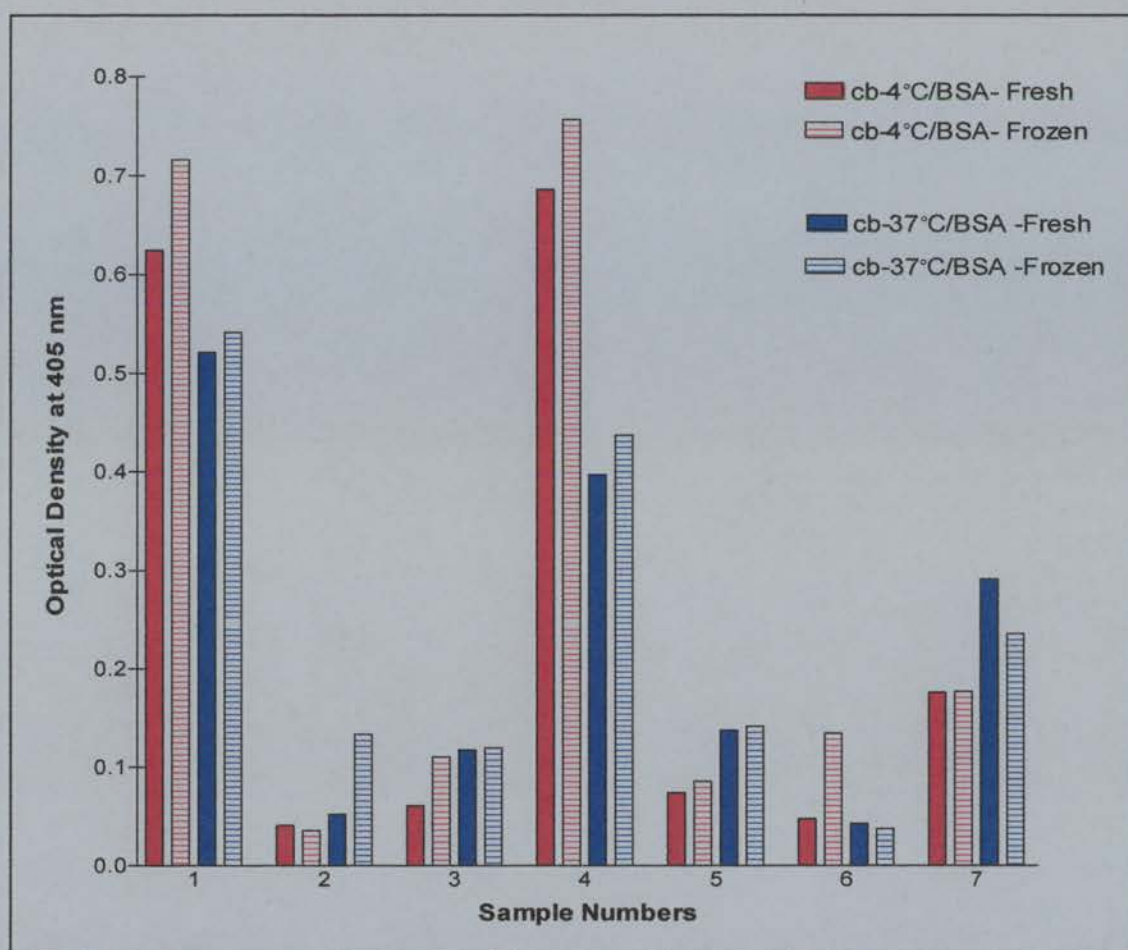


Figure 3.6a: Comparison of coating temperature & storage conditions. Seven serum samples, 2 of which were from IgA EmA positive untreated CD patients (sample numbers 1 and 4), were diluted at 1/50 and the OD responses were compared. The OD responses were higher after coating at 4°C for the 2 EmA positive samples and there was no reduction in OD responses after freezing at -20°C for 24 hours.

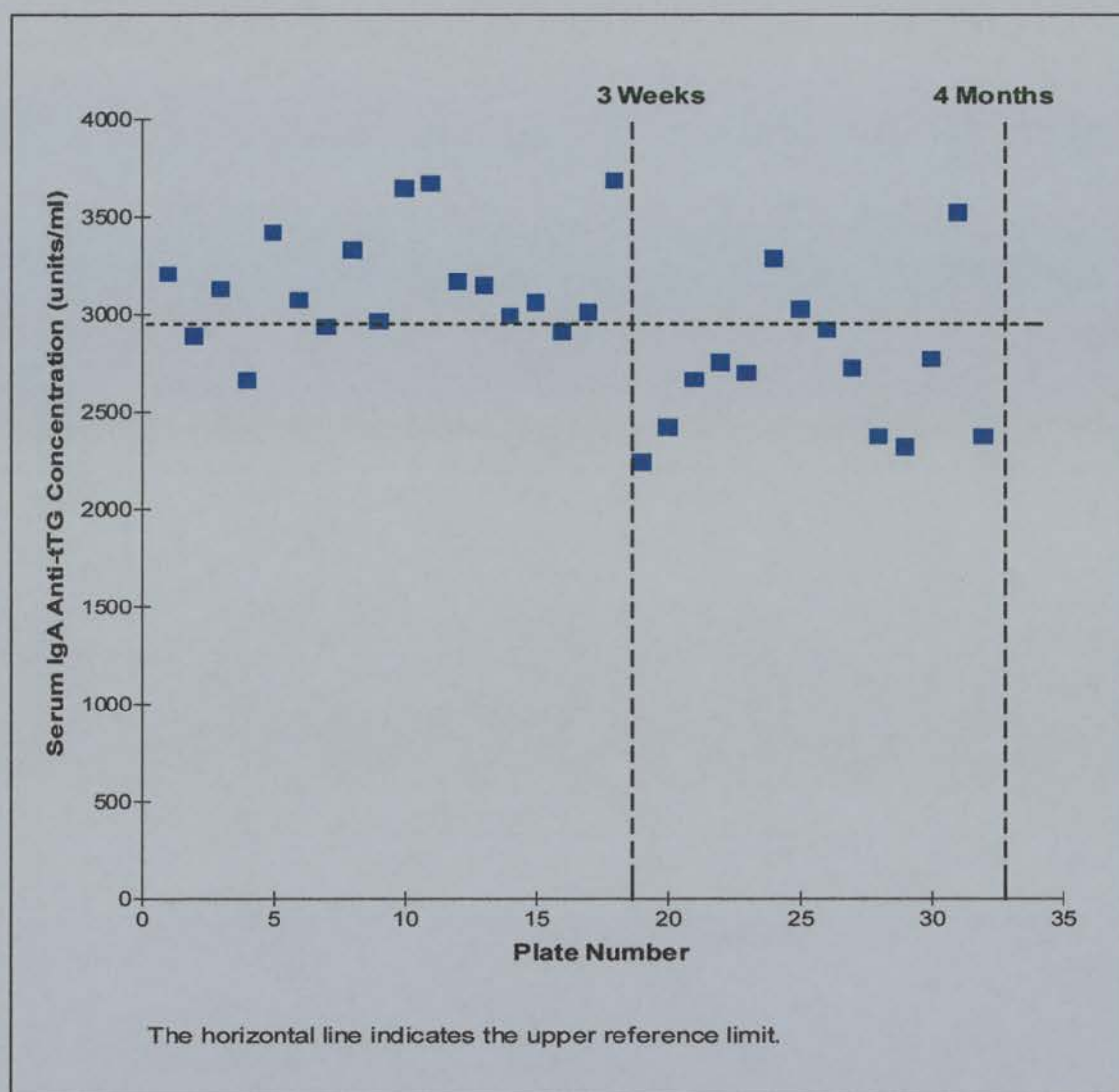


Figure 3.6b: The stability of plates after prolonged storage at -20°C was studied by plotting the OD responses of the HiQC used on each plate for a single batch stored for up to 4 months. The OD readings are shown to decline sharply after plates are stored for more than 3 weeks and therefore plates should not be used after this duration.

3.1.11 Calculation & Acceptance of Results

Plates were read using a Dynatech MR5000 Microplate reader that consisted of a computer terminal with Revelations software, microplate reader and printer. The absorbance was measured at 405nm. The OD readings of the reference standard were plotted using a quadratic regression equation without data extrapolation. The program plotted the best curve fitting through the mean of the ODs of each standard dilution. The top serum reference standard was designated an arbitrary value of 25000 units/ml. The ODs of test samples were compared to the standard curve, and the program calculated the sample concentrations. The lower limits of detection in serum and WGLF were calculated to be 300 units/ml and 15 units/ml respectively. If the OD of the test serum or WGLF sample exceeded the top reference standard, the sample was doubly diluted further and repeated. If two ODs were close together on the linear part of the curve, the average of these values was taken. The concentrations were rounded off to the nearest 5 units/ml.

Test results were acceptable if the HiQC value lay within ± 2 standard deviations (SDs) of the mean value on 28 consecutive runs of the assay and if the duplicate values differed by no more than 10%. This HiQC range was calculated to be between 2010 units/ml to 3730 units/ml. Assays with a standard curve with an R-squared value of >0.98 and a blank OD value of <0.09 were technically acceptable.

3.1.12 Performance Characteristics

During the developmental stages of this project, it was reported that the protein content of the guinea pig liver tTG (Sigma T5398) might vary from batch-to-batch (Maki, 1997a). In the early stages of the work, a record of the lot number of each bottle of tTG was not kept, however this was done in the latter stages. Where possible, several bottles of the same lot number were purchased to reduce the contribution of this variable to the

assay performance. The batch-to-batch variation in the concentration of the HiQC is shown in **Figure 3.7** in which the horizontal dotted lines represent the calculated HiQC range.

The CV, defined as the SD as a percentage of the mean, was calculated as a measure of the precision of the assay. The inter-assay CV was measured to give an estimate of the variability of the assay between runs and was determined by using the HiQC concentrations on 25 consecutive runs of the assay. The intra-assay CV was a measure of the variability of concentrations of a single sample at one dilution at different locations on the plate. Acceptable levels for the inter-assay and intra-assay CVs should be less than 10-15%. The inter-assay CV in this assay was 9.1%. The intra-assay CVs were 12.3% and 11.8% using serum samples above and below the reference range respectively.

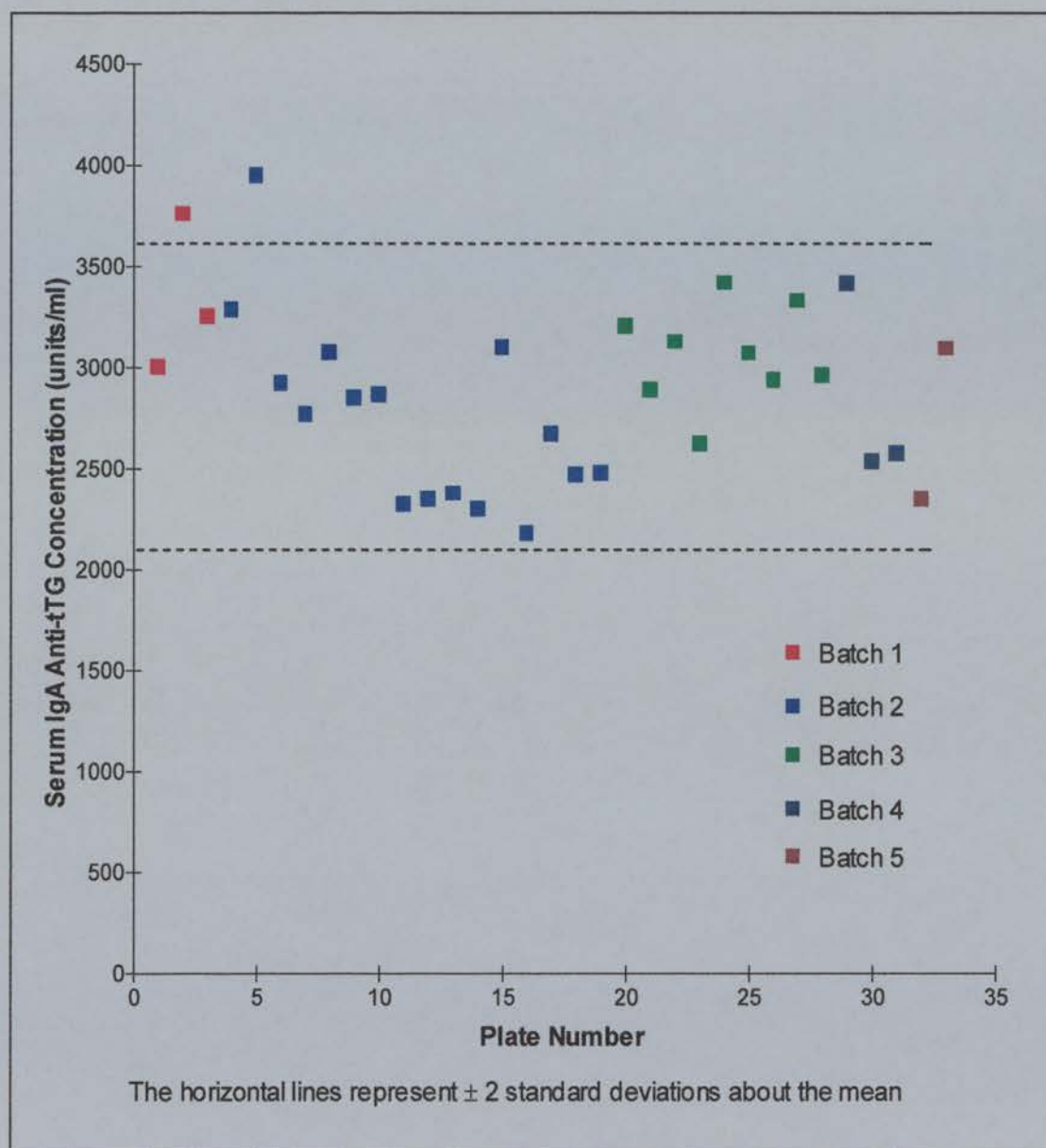


Figure 3.7: Batch-to-batch variation of the HiQC in tTG-coated plates

3.1.13 Requirement for Calcium in Coating Buffer

Although calcium is known to produce a conformational change in tTG which is required for its catalytic activity (Folk, 1972), the requirement for calcium in the coating stage of the ELISA has been questioned. In the ELISA, tTG is used as an antigen and not as an enzyme. The addition of calcium to the assay was therefore studied. These experiments were done in the latter part of the study prompted by correspondence from Dr. M. Maki in Finland in advance of his published findings using the calcium-modified assay (Sulkanen et al. 1998b). In brief, the method described by Maki *et al.*, used plates coated with 100 μ l/well of a Tris/HCl coating buffer (0.05M Tris-HCl, 0.15M NaCl, 5 mM CaCl_2 , and pH 7.5). Incubation was for 2 hours at 37°C. The plates were blocked with a dilution buffer (0.05M Tris-HCl, 0.15M NaCl, 0.01M EDTA, 0.1% Tween 20, pH 7.4) for 1 hour at RT.

Firstly, to investigate if calcium activation was required to increase binding of the tTG antigen to the plate, plates were coated with 100 μ l/well of PBS only and with 10 μ g/ml of tTG in PBS. Other plates were coated with 10 μ g/ml of tTG in CB with and without the addition of 5 mmol calcium chloride (CaCl_2 , Sigma). All of these plates were also incubated overnight at 4°C, were blocked with 1% BSA. In addition, the some of the CB plates with and without added calcium were blocked with 1% Hb instead of BSA. The same 3 serum samples previously used were doubly diluted from 1/25 in diluent solution and 100 μ l/well (in duplicate) were added to the plates. After colour development and reading, the findings showed that there was no reactivity when serum was incubated on plates coated with PBS only. Reactivity was observed only when tTG was added to the coating solution (**Figure 3.8a**). This observation should also hold true for the CB coating buffer. It was found that in the BSA and Hb blocked plates, the addition of calcium increased and reduced the ODs of 2 of the 3 samples tested respectively (**Figures 3.8b & 3.8c**). Although these findings indicated that the addition

of calcium to the BSA blocked method may increase the OD responses of test samples, it showed that calcium was not required to facilitate tTG binding to the plate.

Comparison of OD responses was also made between Maki's method and the CB coated, BSA blocked plates with and without the addition of 5 mmol CaCl₂ using the same 3 serum samples. These samples were doubly diluted from 1/25 and 100 µl/well was added to the plates (in duplicate). Colour development was the same for both methods. This showed that the ODs recorded using the Maki method were higher than those using the CB system with or without the addition of calcium, but were all in the same sequence (**Figures 3.9a & 3.9b**).

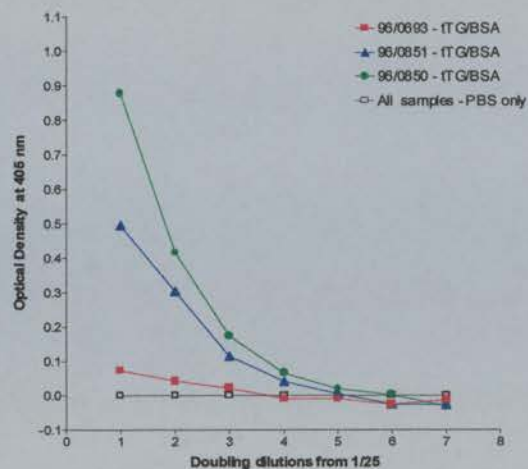


Figure 3.8a:
OD responses to tTG of test samples on BSA blocked plates

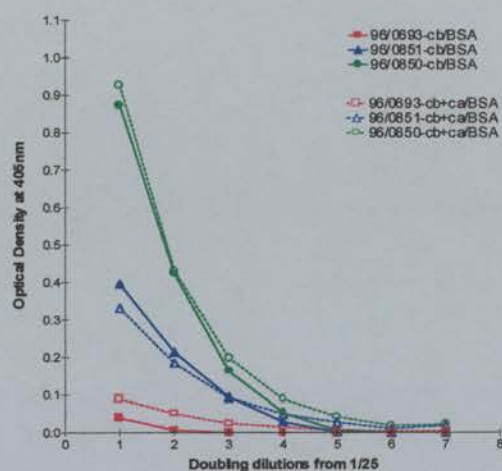


Figure 3.8b:
Comparison of OD responses to tTG with & without calcium on BSA-blocked plates

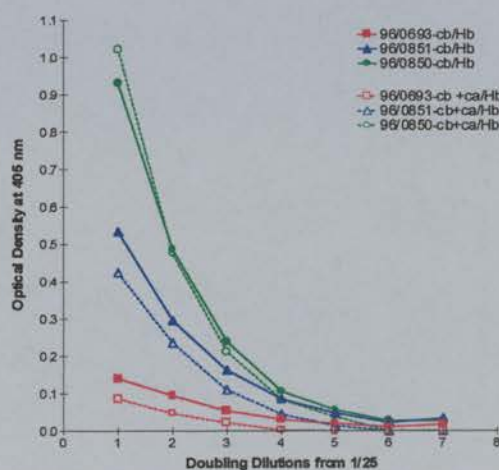


Figure 3.8c:
Comparison of OD responses to tTG with & without calcium on Hb-blocked plates

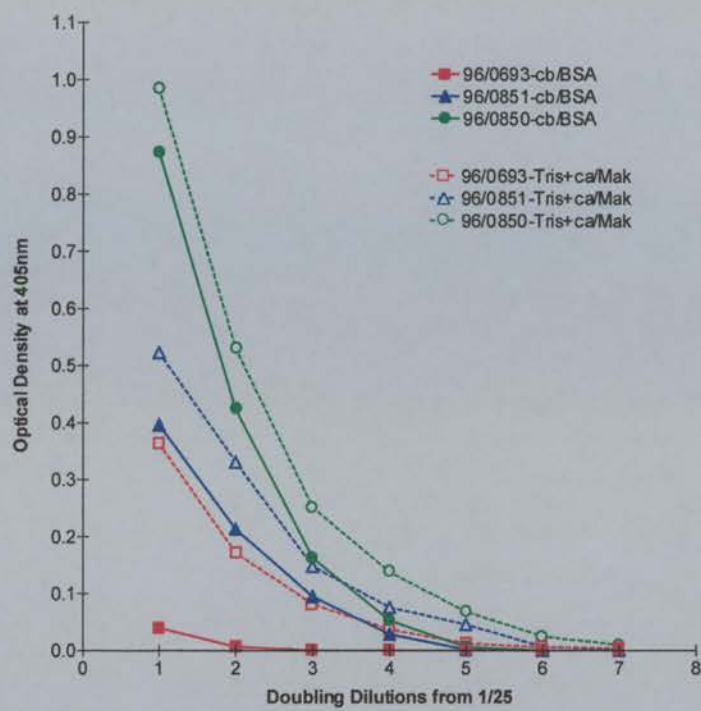


Figure 3.9a:
Comparison of OD responses to fTG without calcium with the Maki method

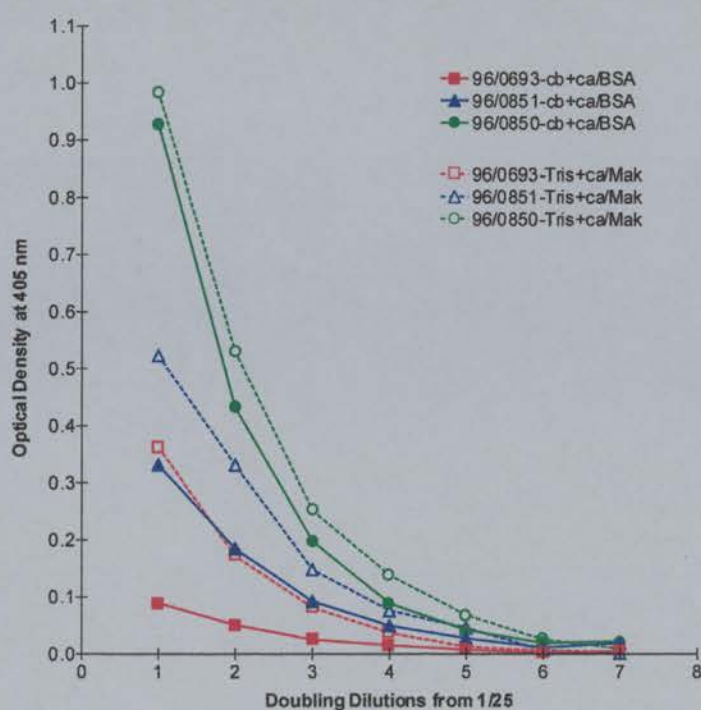
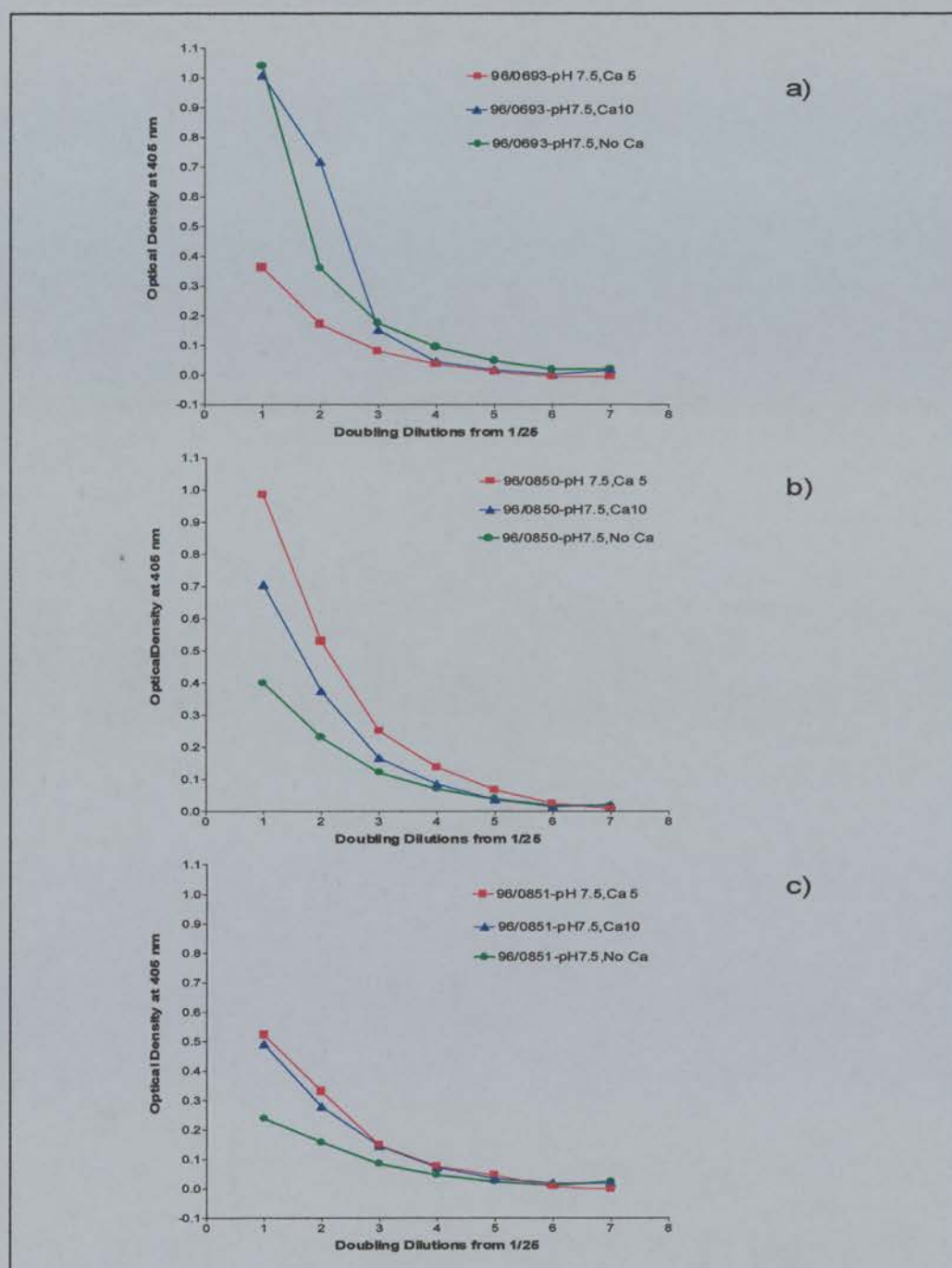


Figure 3.9b:
Comparison of OD responses to fTG with calcium with the Maki method

The optimum activation conditions for guinea pig liver tTG are known to be a calcium concentration of 10 mmol/l and a pH of 8.5 (Bruce et al. 1985). However, Maki did not use this calcium concentration or pH. The effect of a change in the CaCl_2 concentration on the OD responses of the 3 samples were studied in the Maki method when the CaCl_2 concentration was increased from 5 to 10 mmol/l or if CaCl_2 was omitted. This showed that the OD responses reduced in 2 of the 3 samples when there was an increase in the CaCl_2 concentration from 5 to 10 mmol/l and were reduced further when no CaCl_2 was added (**Figures 3.10a-c**). The effect of a change in the coating pH from 7.8 to 8.5 was also studied using the same 3 serum samples. This showed that the ODs were reduced in 2 of the 3 samples if the coating pH was increased from 7.5 to 8.5 (**Figures 3.11a-c**). This showed that the CaCl_2 concentration and pH used in the Maki method were better than the theoretical optimum activation conditions for the enzyme.



Figures 3.10 a-c: Effect on OD responses of a change in calcium concentration to tTG in 3 serum samples

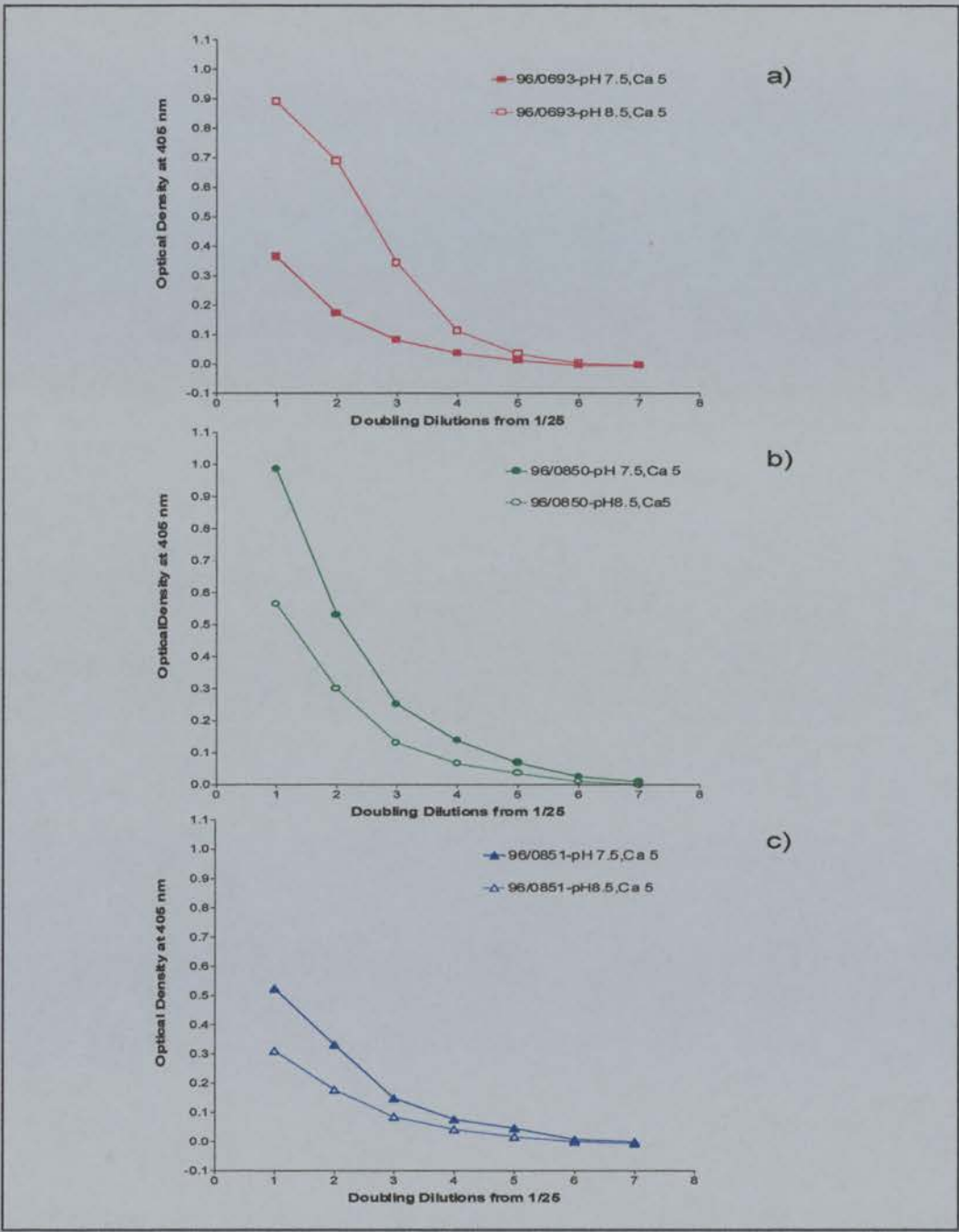


Figure 3.11a-c: Effect of a change in the coating pH on the OD responses to tTG in 3 serum samples

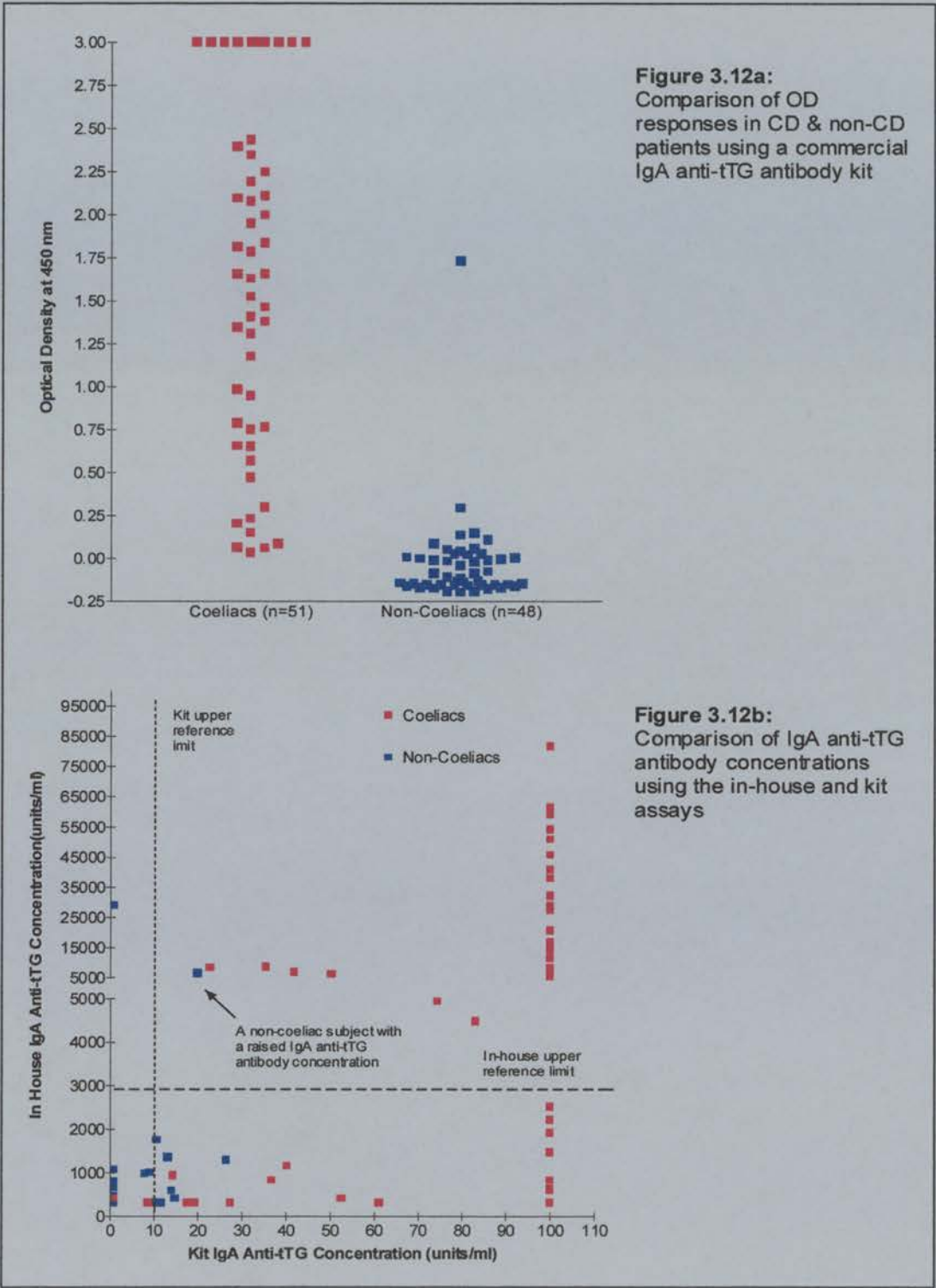
3.2 Method Validation of the IgA Anti-tTG Antibody ELISA

3.2.1 Comparison of IgA Anti-tTG Antibody ELISA with a Commercial Kit

The in-house ELISA was compared with a commercial kit (Genesis Diagnostics, Product No. GD70, Cambridgeshire, UK). According to the accompanying written instructions, the IgA anti-tTG kit used a 96-well plate pre-coated with calcium-activated guinea pig liver tTG. The kit contained pre-diluted reference standard, positive and negative control sera, sample diluent, wash buffer, enzyme conjugate (peroxidase conjugated rabbit anti-human IgA), TMB enzyme substrate, a stop solution and an incubation bag. Test samples were diluted at 1/100 in sample diluent, dispensed into the appropriate wells and incubated for 30 minutes at RT. The plate was 3 times washed using a manual washer. 100 µl of enzyme conjugate was added to each well and incubated for 30 minutes at RT. After a further wash cycle, 100 µl of enzyme substrate solution was dispensed into each well and incubated for 10 minutes. Following this, 100µl of stop solution was added to each well and the ODs were read at 405 nm. A Revelations program on the ELISA reader was developed using a four-parameter, log/logistic curve fit. Users were advised that the positive and negative reference ranges provided should be validated in their own laboratories.

Using 3 kits, IgA anti-tTG antibodies were measured in 51 untreated CD patients and in 48 non-CD patients with normal duodenal histology. All samples were diluted at 1/100 in the sample diluent provided. At this dilution, the ODs of 11 CD samples exceeded that of the top standard, and these samples were designated an OD reading of 3.00 in **(Figure 3.12a)**. It was also noted that many of the non-CD samples had ODs below zero. This was because the average blank values on the plates with the non-CD samples were higher (0.286 and 0.115), than the plate used for most of the CD samples (0.038), although the same reagents were used in all of these plates.

IgA anti-tTG antibody concentrations in the two patient groups were also compared using the in-house and kit assay methods (**Figure 3.12b**). The upper reference limits of normal were 2950 units/ml and 10 units/ml respectively. Serum IgA anti-tTG antibody concentrations using the in-house assay and the tTG kit were raised in 33/51 (65%) and 47/51 (92%) of untreated CD patients and in 2/48 (4%) and 9/48 (19%) of non-CD patients respectively. The serum IgA anti-tTG antibody concentrations using the tTG kit were raised in 14/18 (78%) of the untreated CD patients with normal antibody concentrations using the in-house assay. None of the 4 untreated CD patients with a normal serum antibody concentration using the tTG kit had a raised antibody concentration using the in-house assay. One of the non-coeliac subjects had a raised serum IgA anti-tTG antibody concentration using both assays (**as indicated by arrow in Figure 3.12b**). These findings suggest that the commercial kit is superior to the in-house assay with regard to sensitivity, but is less specific.



3.2.2 Measurement of WGLF IgA Anti-tTG Antibodies using a Serum Standard

Serum from an untreated CD patient was more readily available and was thought to be more stable during storage than WGLF. The volume of serum standard required was also smaller as the serum was diluted at 1/100 whereas the WGLF was diluted at 1/2. To confirm that a serum standard would be a suitable substitute for WGLF, the OD readings of doubling dilutions of the serum standard and 4 WGLF samples from untreated CD patients were compared. This showed that the dilution curves of the serum standard and the WGLF samples were parallel (**Figure 3.13**).

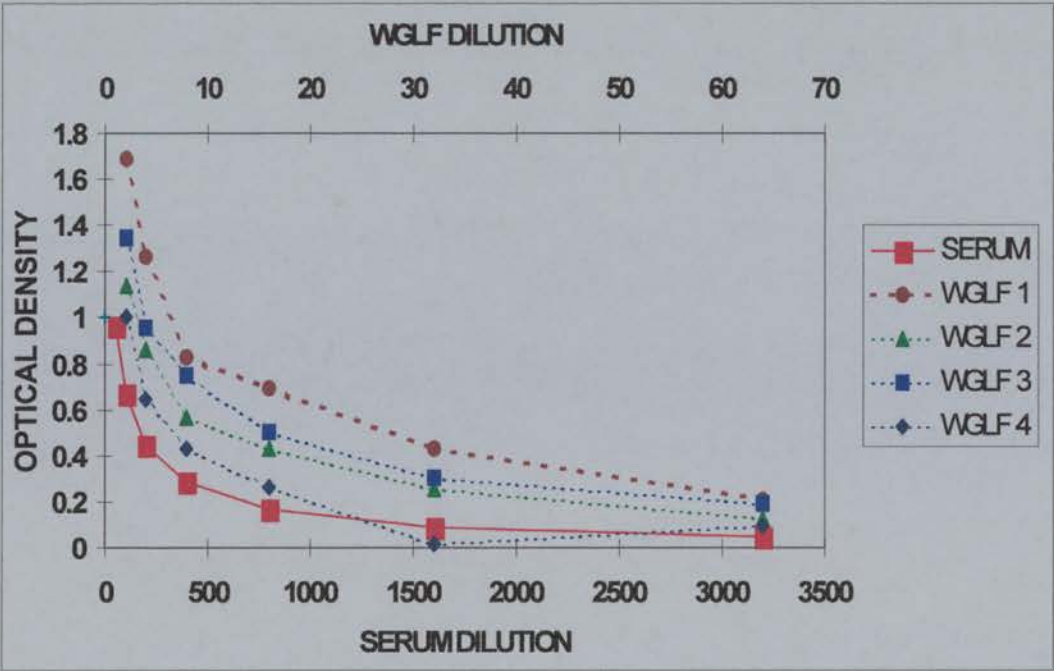


Figure 3.13: Comparison of dilution curves of serum standard & WGLF samples

3.3 Final IgA Anti-tTG Antibody ELISA Method

3.3.1 Specific Reagents

1. **Coating Antigen** - tTG from guinea pig liver (Sigma T5398) was available in dried powder form.
2. **Coating Buffer** - Carbonate-bicarbonate buffer (0.05M, pH 9.6) at 25°C was prepared by dissolving the contents of 10 capsules (Sigma Cat. No. C-3041) in 1 litre of sterile water. The buffer was stored at 4°C for a maximum of 4 weeks.
3. **Phosphate buffered saline** - (PBS, Sigma, P-4417) was prepared by adding 1 tablet to 200 ml of sterile water.
4. **tTG diluent** - was prepared by mixing 1 L of PBS with 0.05% (v/v) Tween 20.
5. **Bovine serum albumin** - (BSA, Sigma, A-7906) was available in dried powder form.
6. **Blocking solution** - consisted of 1% (w/v) BSA in tTG diluent.
7. **Conjugate** - Goat anti-human IgA (α -chain specific) alkaline phosphatase conjugate (Sigma, A-3063).

3.3.2 Procedure in Serum & Whole Gut Lavage Fluid

The guinea pig liver tTG protein content was calculated from the units/mg protein data supplied on each bottle. The entire content of each bottle was reconstituted in coating buffer to a concentration of 10 μ g/ml. A 2.0 unit bottle was sufficient to coat 9 plates. Immulon 2 plates were coated with this coating solution and were incubated overnight (approximately 15 hours) in a covered moist box at 4°C. Plates were washed 3 times with wash solution using the multi-reagent washer and blocked (250 μ l/well) with blocking solution at RT for 2 hours. Plates were then frozen at -20°C until use. Once ready to use, plates were defrosted at RT and the blocking solution decanted, but not washed again.

The serum reference standard was from an untreated CD patient who had given written consent for hepatitis B and HIV testing. A high quality control (HiQC) was from an untreated CD patient with a serum IgA anti-tTG concentration above the reference range. A low QC (LoQC) was from a non-coeliac patient, with a serum IgA anti-tTG within the reference range. Both standard and QC material were aliquotted and stored at -20°C. The serum standard and QC samples were used for both serum and WGLF analysis and were used on all plates. The standard sample was used at a dilution of 1/50 in tTG diluent. Aliquots of Hi and LoQC sera, as well as test sera, were diluted at 1/50 and 1/100 in tTG diluent. Test WGLF samples were diluted at 1/2 and 1/4 in tTG diluent.

Test serum and/or WGLF samples were added to the plate in duplicate and a worksheet was labelled with the corresponding positions and sample numbers as a record. Where possible, paired serum and WGLF samples were analysed on the same ELISA plate. Following an overnight incubation in a moist, covered box at 4°C, the plates were washed. The conjugate, diluted at 1/800 in tTG diluent, was added to each plate. Plates were incubated at RT for 5 hours, washed and then substrate solution was added. The plates were read using the Revelations software on the ELISA reader. Test results were acceptable if the criteria discussed in **section 3.1.11** were satisfied.

3.4 IgG Anti-tTG Antibody ELISA Method Development

3.4.1 Background

The method for the IgG anti-tTG ELISA was adapted from the developed IgA anti-tTG antibody in-house method. The ELISA plate type, antigen substrate, coating buffer, blocking and diluent solutions used were all similar to that used in the IgA anti-tTG antibody method. The conjugate used was a goat anti-human IgG alkaline phosphatase antibody.

3.4.2 Selection of Standard & QC Sera

Serum samples with known IgA anti-tTG antibody concentrations were tested for suitability for use as a standard, Hi and LoQC in the IgG assay. Forty-eight serum samples were tested from untreated and treated CD patients including 2 treated patients with SIgAD and 3 patients with a positive IgG EmA. The standard and QC material used in the IgA anti-tTG antibody assays were also tested for IgG anti-tTG antibodies. All samples were diluted at 1/50 in diluent solution and added to the plate at 100 µl/well in duplicate, and a blank sample was included. IgG conjugate was added at a dilution of 1/800 and colour development was with p-nitrophenylphosphate substrate in DEA buffer. Colour development was monitored using the ELISA reader with a Revelations program. The plate was read after the appearance of a yellow colour change in any of the wells, which occurred after about 7 minutes. Optical densities of the test samples were compared.

The highest average OD readings of duplicate wells (in brackets) were found in 1 of the IgA deficient patients: 98/0781 (1.378), an untreated CD patient: 97/2810 (1.537), the IgA anti-tTG HiQC (1.651), and the 3 IgG EmA positive samples (1.349, 1.652, 1.235).

The average blank value was 0.091. Unfortunately, the IgG EmA positive samples were in too limited supply for use as a standard or QC. Three samples were selected (98/0781, 97/2810, 96/0017) for consideration for use as a standard. These samples were each diluted at 1/50 in diluent and doubly diluted in columns to form standard curves. The OD readings at each dilution were compared (**Figure 3.14a**). The standard curves were also compared using the revelations software on the ELISA reader. Sample 98/0781 produced the best standard curve of the 3 samples tested and it was selected for use as a standard. The IgA anti-tTG antibody concentration was < 300 units/ml in this sample. This patient, who had been on a gluten-free diet for 4 years, gave written consent for hepatitis B and HIV testing and donated a large volume of blood which was centrifuged, aliquotted and stored at -20°C. Two of the 48 samples tested with appropriate OD readings were aliquotted for use as a Hi (97/3158) and LoQC (97/3073).

3.4.3 Optimal Sample Dilution

Optical density readings of test samples fell within the linear segment of the standard curve most frequently with serum samples diluted at 1/25, 1/50 and 1/100. The same serum standard and QC material were used in the WGLF assay, as was performed with the serum IgG anti-tTG ELISA. WGLF samples from 12 untreated CD patients were tested for the presence of IgG anti-tTG antibodies. All samples were doubly diluted from 1/2 to 1/32. None of the samples tested, even at the 1/2 dilution, had detectable antibody levels. No further WGLF samples were analysed.

3.4.4 Optimal Conjugate Dilution

A single serum sample with high OD readings for IgG anti-tTG was selected and doubling dilutions from 1/50 in duplicate columns were performed. IgG conjugate was added at doubling dilutions from 1/100 to 1/3200 to each duplicate columns. The OD readings were compared across the plate with increasing conjugate dilution. This

showed that OD readings plateaued at conjugate dilutions of 1/400 and 1/800, but markedly declined thereafter. An optimal conjugate dilution of 1/800 was selected **(Figure 3.14b)**.

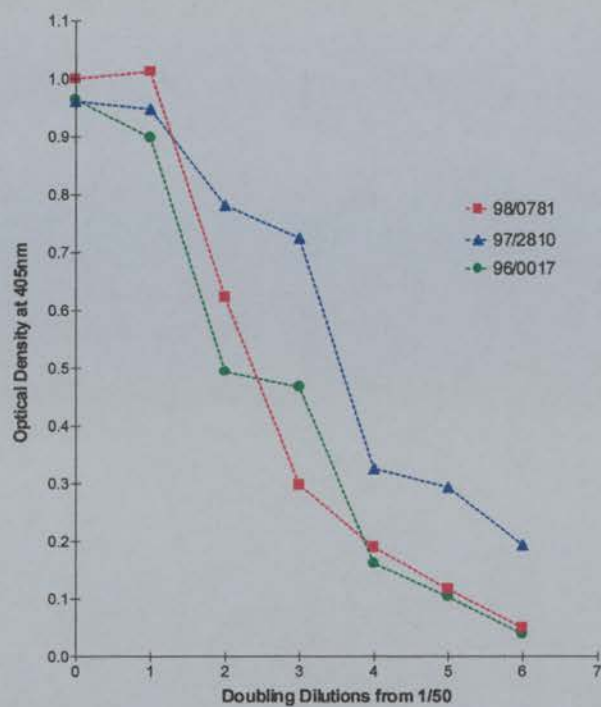


Figure 3.14a:
Comparison of OD
responses to tTG of 3
potential serum standards in
the IgG anti-tTG antibody
ELISA

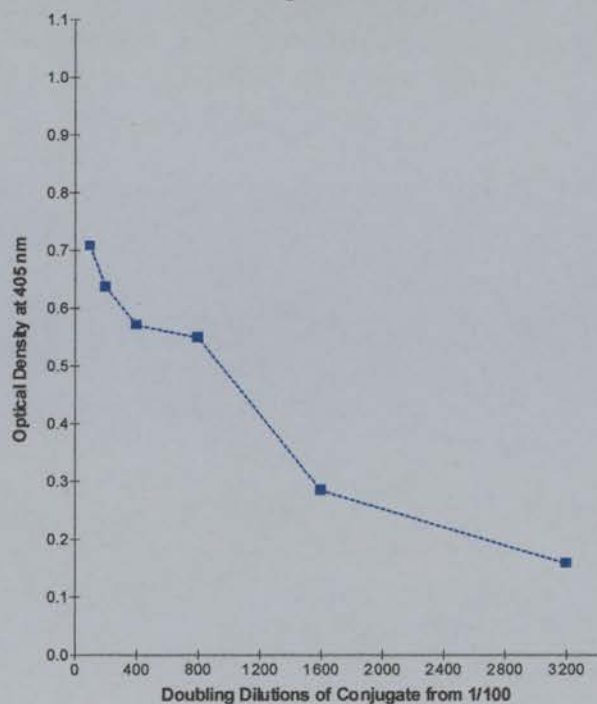


Figure 3.14b:
Determination of the optimal
IgG conjugate dilution

3.4.5 Upper Reference Limit of IgG Anti-tTG in Serum

The upper reference limit for the IgG anti-tTG assay was determined by measuring the antibody concentrations in the plasma of the same 106 anonymous blood donors used for the IgA anti-tTG assay using the non-parametric method described in **section 3.1.6**. An upper reference range of 14910 units/ml was obtained which excluded the 6 highest donor plasma concentrations (**Figure 3.15**). The blood donor with the very high IgA anti-tTG antibody concentration of 31700 units/ml also had a raised IgG anti-tTG antibody concentration of 21400 units/ml.

During the initial stages of development of the IgG anti-tTG assay, sera from 20 unselected untreated CD patients were analysed. The IgA-class anti-tTG, EmA and AGA, and IgG AGA were positive in 14 (70%), 15 (75%), 12 (60%) and 17 (85%) patients respectively. The IgG anti-tTG however was only positive in 2 (10%) of these patients. As IgG anti-tTG antibodies were not detectable in WGLF and the assay was also insensitive, no further development work was undertaken.

3.4.6 Calculation & Acceptance of Results

The revelations program used for the IgA anti-tTG assay was modified. To produce the best standard curve, an R-squared value of >0.98 and a blank value of <0.09 were technically acceptable. Like the IgA assay, the IgG serum reference standard was designated an arbitrary value of 25000 units/ml. The lower limit of detection in serum was 300 units/ml. If the OD readings of the test serum exceeded the top reference standard, the sample was doubly diluted further. The concentration corresponding to the OD lying in the linear part of the standard curve was used as the final test result. If two ODs were close together on the linear part of the curve, the average of these values was taken and the concentration was rounded off to the nearest 5 units/ml. Test results were acceptable if the QC absorbance values lay within ± 2 SDs of the mean value of 9

consecutive runs of the assay and the duplicate values differed by no more than 10%. The HiQC range was calculated to be between 7705 units/ml to 14305 units/ml and the LoQC range was between 3875 units/ml to 7160 units/ml within a single batch of plates.

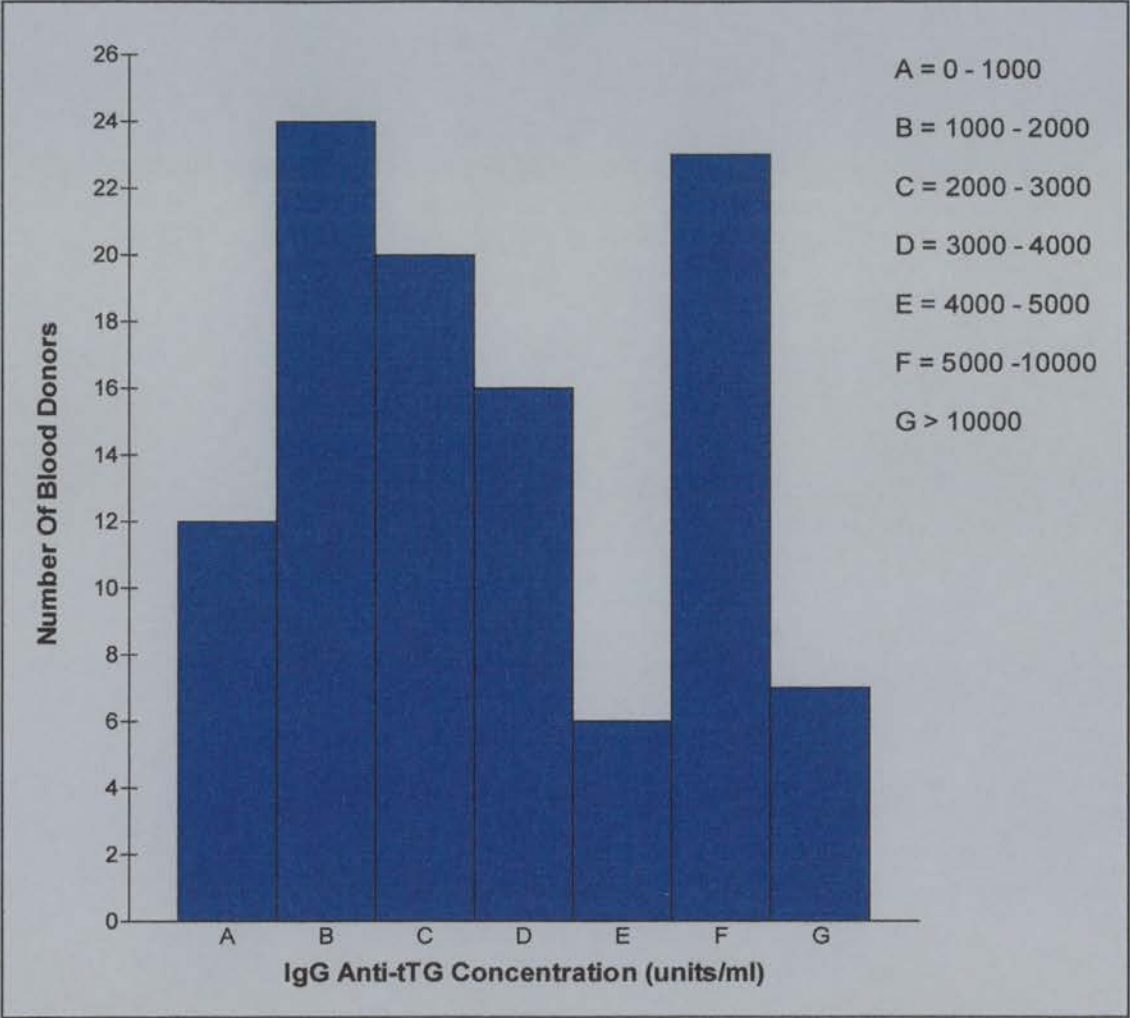


Figure 3.15: Frequency distribution of IgG anti-tTG antibody concentration in blood donors

3.4.7 Performance Characteristics

The concentrations of the HiQC and LoQC samples for each assay performed were recorded to monitor the assay performance. Consecutive runs of the assay showed that there was much more variability in the QC results in the IgG assay than in the IgA assay. This was indicated by the large spread of concentrations about the mean for both the HiQC and LoQC, even in a single batch of plates. The batch-to-batch variation in concentrations was also high with HiQC values in the first plate of consecutive batches ranging from 11500 units/ml in one batch to 23500 units/ml in the next batch. Similar variation was encountered in the LoQC concentration, which ranged from 4070 units/ml in the first plate of a batch to 7950 units/ml in the next batch. For this reason the inter-assay CVs of the HiQC and LoQC samples on 25 consecutive runs of the assay and were both high at 15%. The intra-assay CV's using the HiQC and LoQC samples were 8.8% and 13.9% respectively. This assay was disappointingly less reliable and reproducible than the IgA anti-tTG assay.

3.5 Final IgG Anti-tTG Antibody ELISA Method

3.5.1 Specific Reagents

1. **Conjugate** - Goat anti-human IgG (γ -chain specific) alkaline phosphatase conjugate (Sigma, A-3187)
2. All other reagents were those used in the IgA anti-tTG method.

3.5.2 Procedure in Serum & Specific Reagents

The coating and blocking procedures for this assay were the same as those for the IgA anti-tTG assay. The serum reference standard was from a CD patient with SIgAD. Hi

and LoQC sera were from CD patients with appropriate IgG anti-tTG concentrations with respect to the reference range. The standard was diluted at 1/50 in tTG diluent and added to the plate as previously described to form a standard curve. Hi and LoQC sera, as well as test serum samples, were diluted at 1/25, 1/50 and 1/100 in tTG diluent. The WGLF samples were doubly diluted from 1/2 to 1/32 in tTG diluent. After an overnight incubation and washing, the conjugate was added to the plate at a dilution of 1/800. Plates were incubated for 5 hours at RT and were then washed again. Colour development and monitoring was as previously described for the IgA anti-tTG method. Test results were acceptable if the criteria discussed in **section 3.4.5** were satisfied.

3.6 Discussion

The developmental issues discussed above show that establishing a new ELISA method requires much attention to detail. This includes plate selection, optimal coating, blocking, conjugate and sample dilution, as well as appropriate selection of reference standard and QC material. The stability and storage conditions are also very important. After the investigative steps outlined above, we concluded that the optimal coating concentration of tTG was 10 µg/ml. Methodologically, the most contentious issues were the omission of calcium in the coating solution and use of BSA in the blocking solution.

We found that the addition of CaCl₂ to the CB system led to a reduction in the OD readings of test samples. In retrospect, the likely reason for this observation was the production of calcium bicarbonate, which is not very soluble. Another explanation could be revealed by the finding in rabbits that there were two antigenic sites on the tTG molecule (Fesus and Laki, 1977). In this experiment, rabbits immunised with purified guinea pig liver tTG showed two antibody populations against the enzyme, one which reacted only with the calcium-tTG complex and another which reacted with the inactive

as well as the calcium-tTG complex. These investigators concluded that the calcium-induced conformational changes in tTG gave rise to a specific antibody population so that depending upon whether the inactive enzyme, or the calcium-activated enzyme was used as the antigen, a different enzyme-antibody interaction occurred. The heterogeneity of the antibody population was confirmed using a quantitative precipitin test at a pH of 7.5.

Although this study has not been reproduced in humans, it seems likely that both forms of tTG (intact or calcium-enzyme complex) exists *in vivo*. It would therefore seem more accurate to measure both antibody populations using the inactive enzyme as the antigen as used in our in-house ELISA. In practice however, the Maki system with calcium appeared more sensitive than the in-house ELISA. In view of the potential interaction between calcium and CB, the only way to incorporate calcium into our existing method would be to change pH by substituting the CB with PBS. Alternatively, in future we could change over to the Maki conditions. Neither of these options could be explored during this project, as these findings were only made after a significant number of test sera and WGLF samples had been analysed. Changing the method at that time would have led to difficulties in comparing results obtained using different methods.

With regard to the use of BSA in the blocking solution, we found that both untreated CD patients and non-CD patients may possess anti-BSA antibodies, but the reaction appears to be slow and the concentration to be low. In future, a biologically inert protein, such as Hb, may be preferable. Indeed, we found that the OD readings on plates blocked with Hb were higher than the BSA-blocked plates (**figure 3.3b**), however it was not practical to change the method during the advanced stages of the study.

The commercial IgA anti-tTG kit was more sensitive, but less specific, than the in-house assay. There however were some problems encountered with use of the commercial kit. The instructions enclosed in the first trial kits purchased stated an incorrect incubation time for the substrate of 30 minutes. An error was suspected when the top 2 or 3 standard ODs were > 2.000, instead of 1.00 indicating that the plates had over-developed. When the company was contacted by telephone to discuss this, the representative explained that there was a clerical error and that the incubation time should have stated only 10 minutes. In compensation, 2 new kits were dispatched, and the time-consuming experiments had to be repeated. The other main problem was an omission in the instructions explaining how the plates should be read using Revelations software. Another telephone call clarified the program used by the company. The main disadvantage of the use of a commercial kit for routine serological screening is the cost. The reference range would also have to be locally validated.

The IgG anti-tTG ELISA was insensitive and was poorly reproducible. IgG anti-tTG antibody concentrations were raised in the IgA deficient patients, despite their treatment with a GFD. These findings are consistent with the observation that EmA antibodies are predominantly of the IgA class (Chorzelski et al. 1984) and that the IgG EmA test produces more false positives (Unsworth, 1996). The use of IgG EmA is only justified in IgA deficient patients where it has been shown to be sensitive (Sulkanen et al. 1998a). Likewise, the IgG anti-tTG assay is not useful in screening, but may have a role to play in these selective patients. Raised concentrations despite prolonged treatment with a GFD would also suggest that these antibodies are not useful in monitoring gluten withdrawal or dietary compliance.

In addition, IgG anti-tTG antibodies could not be detected in WGLF. A likely explanation is because the number of IgA producing cells in small intestinal mucosa of untreated CD patients far exceed the number of IgG producing cells (Colombel et al.

1990; Jonard et al. 1984), and consequently, IgA is the predominant Ig in mucosal secretions (Gaspari et al. 1988). Although the serum reference was able to demonstrate WGLF IgA anti-tTG antibodies, it may have been too concentrated to identify the IgG class. If a serum reference was used, it probably should have been diluted more than the initial 1/50 dilution used. Alternatively, a WGLF reference standard could have been selected. I did not find this an attractive option as a large volume of material would have been required and it would have made comparison with the paired serum antibody concentrations less valid.

The steps taken to develop the IgA and IgG anti-tTG ELISA allowed me to understand the difficulties involved in establishing a new assay. I learned some hard lessons along the way in particular, not to share standard or QC material with routine laboratory assays.

CHAPTER 4: MATERIALS & METHODS

4.1 Specimen Collection & Processing

4.1.1 Serum

Approximately 30 ml of venous blood was withdrawn from each patient with suspected CD using a monovette needle and the samples were allowed to clot at RT for 1 hour. Each blood sample was centrifuged at 2500 rpm (1400 g) for 10 minutes and the serum was separated into 2 ml storage tubes, which were labelled for identification. One sample was stored at -20°C for routine analysis (IgA AGA and EmA) and 4-8 samples were stored for research purposes at -70°C for future use.

4.1.2 Whole Gut Lavage Procedure

The WGL procedure used in this study was based on a previously described method (Gaspari et al. 1988) that was adapted by Dr. S. O'Mahony. The WGL solution was a polyethylene glycol (PEG) based electrolyte solution which was commercially available as Klean-Prep[®], (Norgine, Oxford, UK). Each sachet contained 59g PEG with a molecular weight of 3350; 1.45 g NaCl; 1.63 g NaHCO₃; 5.68 g Na₂SO₄; and 0.75 g KCl (BP). One sachet was dissolved in a litre of drinking water to give an osmolality of 260 mosm/L. After an overnight fast, the patient drank the gut lavage solution aiming for a rate of 250 ml every 15 minutes. This was supervised and monitored by an experienced nurse. All liquid stools and faecal stained fluids passed per rectum were discarded. The first clear sample passed per rectum, referred to as WGLF, was collected. The volume of WGLF required depended on the number and nature of assays planned, but generally 20-30 ml sufficed for most routine use.

4.1.3 Whole Gut Lavage Fluid Collection & Processing

For all of the assays used, protease inhibitors were added to the WGLF to reduce the digestion of luminal antibodies and proteins by bacterial proteases. The WGLF was processed within 10-15 minutes of collection to prevent the degradation of unstable proteins. The WGLF sample was filtered through Whatman GF/A 12.5 (Whatman, International Ltd, Maidstone, England) glass microfibre filters and 5ml of the specimen was mixed after each sequential addition as follows (final concentrations are given in brackets):

1. 0.5 ml (80 µg/ml) Soya Bean Trypsin Inhibitor in phosphate buffered saline (PBS)
 2. 0.28 ml (15 mM) Sodium Ethylene Diamine Tetraacetic acid (EDTA BDH Cat. No. 10093) in PBS
 3. 0.12 ml (2 mM) Phenyl Methyl Sulphonyl Fluoride (Sigma Chemical Co. Ltd, Poole, Dorset Cat. No. 7626) in 95% ethanol
 4. 0.06 ml (1 mM) Sodium azide and leave for 2 minutes
 5. 0.3 ml (5% v/v) Newborn Calf Serum (Sigma Cat. No. N4762-heat inactivated)
- Aliquots of processed WGLF (250 - 1000 µl) were stored at -70°C for later analysis.

4.2 ELISA Assays

4.2.1 Common ELISA Reagents & Materials

1. **Sterile Water** - 1 litre containers (stored at 4°C), Baxter Healthcare, Code F7114.
2. **Glass Distilled Water**
3. **Sodium Chloride** (0.9% w/v, NaCl) - 1 litre containers (stored at 4°C), Baxter Healthcare, Code F7124.
4. **Tween 20** - (Polyoxyethylene-Sorbitan Monolaurate) was obtained from Sigma Cat. No. P-1379.

5. **Coating Buffer** - Carbonate-bicarbonate buffer (0.05M, pH 9.6) at 25°C was prepared by dissolving the contents of 10 capsules (Sigma Cat. No. C-3041) in 1 litre of sterile water. The buffer was stored at 4°C for a maximum of 4 weeks.
6. **Wash Solution** - was prepared by adding and mixing 500 µl of Tween 20 to 1 litre of 0.9% NaCl. The solution was stored at 4 °C and was suitable for either manual washing or was used in the Dynatech MRW ELISA automatic washer.
7. **Adult Bovine Serum** - was purchased from Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire, ML8 5ES (Product No. S026-220) in 20 x 20 ml aliquots and kept frozen at -20°C. An aliquot was thawed and filtered through a 0.22 µm filter before use.
8. **DEA Buffer** - Two litres of DEA substrate was constituted in a semi-quantitative flask by adding 500 ml of DEA (BDH Laboratory Supplies, Poole, Analar Reagent Product No. 10393 4J) to 0.51g magnesium chloride ($\text{Mg Cl}_2 \cdot 6\text{H}_2\text{O}$), 1.0 g sodium azide (NaN_3) and 1 litre of sterile water. The pH was adjusted to 9.8 with 6N hydrochloric acid (HCl) and sterile water was added to make up the final volume of 2 litres. The solution was stored in 1 litre containers at 4°C.
9. **Disodium P-Nitrophenylphosphate Hexahydrate** - 5 mg tablets were obtained from Sigma (200 tablets/bottle, Product No. 104-105) and were stored at -20°C.
10. **Substrate Solution** - Fresh substrate was constituted approximately 30 minutes before required by dissolving 1 tablet of p-nitrophenylphosphate per 5 ml DEA buffer and mixed thoroughly.
11. **Immulon 1 Binding Plates** - Flat-bottomed microtiter plates (12.8 cm x 8.6 cm), 96-well protein binding polystyrene, were obtained from Dynex Technology, Chantilly, VA (Cat. No. 3355).
12. **Immulon 2 High Binding Plates** - Flat-bottomed microtiter plates (12.8 cm x 8.6cm), 96-well plate of irradiated protein binding polystyrene, were obtained from Dynex Technology, Chantilly, VA (Cat. No. 3455).

4.2.2 Instrumentation

1. **Multi-Reagent Washer** - (Dynatech Laboratories, Daux Road, West Sussex, UK) was programmed for 3 wash cycles.
2. **Rotamixer** - (Hook & Tucker Instruments Ltd., Croydon, England) was used to mix serum and whole gut lavage samples before and after dilution.
3. **IKAMAG RH Stirrer** - (Janke & Kunkel, IKA, Laboratechnik, Staufen) with magnetic stirrer was used to stir and dissolve reagents.
4. **Denley Wellmixx 1** - 1 or 2 ELISA plates can be placed onto the platform and shaken automatically to hasten colour development.
5. **Dynatech MR5000 Microplate Reader** - consisted of a computer terminal with Revelations software, printer and microplate reader.

4.2.3 IgA Anti-Gliadin Antibody ELISA

4.2.3.1 Specific Reagents

1. **Coating antigen** - crude gliadin extract from wheat gluten (Sigma, G3375).
2. **Diluent** - 0.9% NaCl, 1% (v/v) adult bovine serum, 0.05% (v/v) Tween 20.
3. **Conjugate** - Goat anti-human IgA (α -chain specific) alkaline phosphatase conjugate (Sigma, A-3063).

4.2.3.2 Procedure in Serum & Whole Gut Lavage Fluid

ELISA methods for the measurement of IgA AGA antibodies in serum and WGLF have been previously developed and validated in this laboratory (O'Mahony et al. 1991). Immulon 2 plates were coated with 100 μ l/well of 25 μ g/ml gliadin in coating buffer. The gliadin was difficult to dissolve and overnight mixing was required. Plates were incubated for 5 hours at RT and then washed. The plates were then blocked with 250 μ l/well of diluent and incubated for 2 hours at RT. Several plates were prepared and frozen at -20°C in an airtight box until use. Plates stored in this manner were stable for

at least 3 months (J. Bode, personal communication). Plates were defrosted at RT before proceeding to the next stage and the diluent was decanted and blotted dry.

The reference standard for both the serum and WGLF IgA AGA analysis was serum from an untreated CD patient with known positive serology and was designated an arbitrary concentration of 100 units/ml. The standard was diluted at 1/620 in diluent, and was added in doubling dilutions to the plate to produce a standard curve. Hi and LoQC sera, and test serum samples were diluted at 1/100. Test WGLF samples were diluted at 1/2 and 1/4. Further dilutions were performed for high samples. A worksheet was prepared to mark the sample positions. Plates were incubated overnight in a moist covered box at 4°C. After washing, a 1/1000 dilution of conjugate in diluent was added and the plates were incubated for 5 hours at RT. The substrate solution was added after washing again, and colour development was monitored on the ELISA reader using a Revelations program designed by Mr. John Bode. The absorbance endpoint for IgA was when the top standard read an OD of 1.00.

Test results were technically acceptable if the QC concentrations were within ± 2 SDs of the mean of a series of previous runs and the duplicate values differed by no more than 10%. The within batch CVs at 20 and 90 units were 9.4% and 7.5% respectively. The between batch CVs at 100 units and 26.5 units were 12.1% and 18.1%. The normal reference range for serum IgA AGA had previously been established at between 0-30 units/ml (J. Bode personal communication). The upper reference limit of WGLF IgA AGA of 5 units/ml was determined by taking the 95th percentile of the results for 49 patients with an immunologically normal gut. These patients included 36 patients with irritable bowel syndrome and 13 healthy volunteers.

4.2.4 IgG Anti-Gliadin Antibody ELISA

4.2.4.1 Specific Reagents

1. **Conjugate** - Goat anti-human IgG (γ -chain specific) alkaline phosphatase conjugate (Sigma, A-3187)
2. All other reagents were similar to those used in the IgA AGA method.

4.2.4.2 Procedure in Serum

This procedure was similar to the IgA AGA assay with the following alterations. The serum reference standard was doubly diluted from 1/1200 in diluent, and the Hi and LoQCs were doubly diluted from 1/200 to 1/1600. Test samples were diluted at 1/200 and 1/400 in diluent. The conjugate was diluted at 1/750. Colour development was monitored using a Revelations program designed by Mr. John Bode. The absorbance endpoint for IgG was when the 2nd standard reached an OD of 1.00. The within batch CVs at 26 units and 117 units were 9.9% and 5.9% respectively. The between batch CVs at 92 units and 162 units were 14.9% and 13.2% respectively. The normal reference range for serum IgG AGA had previously been established at between 0-45 units/ml (J. Bode personal communication).

4.2.5 Total IgA ELISA in Whole Gut Lavage Fluid

4.2.5.1 Specific Reagents

1. **Coating Antigen** - Rabbit anti-human IgA (Dako, A0262)
2. **Standard** - Reagent grade IgA purified from human pooled colostrum (Sigma, I0633)
3. **Diluent** - 0.9% NaCl, 1% (v/v) adult bovine serum, 0.05% (v/v) Tween 20
4. **Conjugate** - goat anti-human IgA (α chain specific) alkaline phosphatase conjugate (Sigma A3063)

4.2.5.2 Procedure

The method for the measurement of total IgA in WGLF was previously developed in our laboratory (O'Mahony et al. 1991). Immulon 1 plates were coated with 100 µl/well of coating antigen diluted to 1/600 in coating buffer and were incubated overnight (at least 16 hours) at 4°C in a moist covered box. The plates were then washed and blotted dry. Plates were blocked with diluent incubated for at least 1 hour at RT.

The reference standard was reconstituted to 210 µg/ml, aliquotted and stored at -20°C. Each aliquot was thawed only once and diluted to a concentration of 1 µg/ml in diluent. The standard was added in doubling dilutions in the first two columns of each plate. A blank sample and fresh aliquot of a HiQC was doubly diluted from 1/100 to 1/6400 and included on each plate. Test WGLF samples (filtered processed) were doubly diluted (in duplicate) from 1/100 to 1/6400 in diluent and 100 µl/well were added to the plates. The plates were then incubated overnight at 4°C. After a further wash cycle, conjugate was added at a dilution of 1/10 000 in diluent and 100 µl was added to each well. The plates were incubated for 3 hours at 24°C and then washed. Substrate solution was added and the colour development was monitored on the ELISA reader at 405 nm.

4.2.6 Total IgG ELISA in Whole Gut Lavage Fluid

4.2.6.1 Specific Reagents

1. **Coating antigen** - Goat anti-human IgG (Fc- specific), (Sigma, I-2136)
2. **Reference standard** - pooled human serum (SPS-01) Batch 98-1, 10.59g/l was obtained from the Supra-regional Protein Reference Unit, Sheffield, UK.
3. **Diluent** - 0.9% NaCl, 1% (v/v) adult bovine serum, 0.05% (v/v) Tween 20
4. **Conjugate** - Goat anti-human IgG (γ-chain specific) alkaline phosphatase conjugate (Sigma, A-3187)

4.2.6.2 Procedure

This assay was originally developed by Dr. S. O'Mahony (O'Mahony et al. 1991) and was performed as part of the routine laboratory service. Immulon 1 plates were coated with 125 µl/well of IgG coating antigen diluted to 1/5000 in coating buffer. Following an overnight incubation at 4°C in a moist covered box, plates were washed. They were then blocked with 250 µl/well of diluent for a minimum of 1 hour at 24°C. After the diluent was decanted and the plate was blotted dry, 125 µl/well the reference standard, diluted at 250 ng/ml, was added in doubling dilutions to the plate to form a standard curve. A HiQC sample, obtained from a Hepatitis B/HIV negative patient with a raised WGLF IgG, was diluted at 1/25 in diluent as were WGLF samples. After mixing diluted WGLF samples, 125 µl/well of each sample was added in duplicate columns, and doubling dilutions were performed. The plates were then incubated in a covered moist box overnight at 4°C. After a washing cycle, 125 µl of the IgG conjugate diluted at 1/5000 was added to each well and incubated in a covered moist box for 3 hours at RT. Colour development was monitored on the ELISA reader until the top standard reached an OD of 1.0.

Results were calculated by creating a standard curve of absorbance readings against concentration of Ig at each dilution of the standard using a Prism software program. The linear portion of the standard curve was used to calculate the linear regression line for these standards. The correlation coefficient for the standard curve was checked and was acceptable if greater than 0.985. Using the linear regression equation, the concentration of the QC and each sample at several dilutions were calculated. Sample results were acceptable if QC values fell within 95% of the current QC mean value. Samples must have 2 or more points parallel to the standard curve and a mean of the concentrations at these parallel points was taken as the result. Samples with absorbances above those of the standard curve were repeated at a starting dilution of 1/250. Results were reported to the nearest whole number, and very high results, which

required further dilution were reported to the nearest 10 µg /ml. The between batch CV for WGLF total IgG was 12.3% at 20 µg/ml. The normal reference range for WGLF total IgG of between 0-10 µg/ml was previously established by Dr. G. Brydon, and an elevated WGLF IgG >10 µg/ml has been shown to be characteristic of active IBD (Brydon et al. 1993; Choudari et al. 1993).

4.3 Albumin in Whole Gut Lavage Fluid

4.3.1 Specific Reagents

1. **PEG Reagent** - 40 g polyethylene glycol 6000 (biochemical grade), 6 g Tris, 2 g Tween 20, 1 g sodium azide dissolved in 800 ml of distilled water, adjusted to pH 7.0 with dilute HCl, and made up to a final volume of 1 litre with distilled water.
2. **Diluent** - 0.9% NaCl, 60 g PEG 3350 and 1 g sodium azide in 1 litre distilled water.
3. **Sheep Anti-human Albumin** - (SAPU - Code S034-205)
4. **Antibody Reagent** - Dilute sheep anti-human albumin serum 1 in 40 with the PEG reagent
5. **Standard reference serum** - (SPS-01) Batch 98-1, 39.6 g/l was obtained from the Supra-regional Protein Reference Unit, Sheffield, UK.

4.3.2 Instrumentation

1. **Gilson Dilugil** - Anachem, Luton, Beds, UK.
2. **Spectrophotometer** - UV/VIS Kinetics Spectrophotometer, PU 8610, Philips.

4.3.3 Procedure

This method was developed in our laboratory by Dr. G. Brydon (Brydon et al. 1993), and the assay was performed as part of the routine laboratory analysis. The assay principle was that human albumin, after interacting with its specific antibody, formed insoluble immunocomplexes which precipitate in the presence of PEG. If the antibody

was present in large excess, these precipitates produced a turbidity, which was related to the concentration of albumin in the sample. The turbidity was spectro-photometrically measured at a wavelength of 340nm. Absorbance readings obtained by assaying calibration standards were used to generate a standard curve, from which the concentration of albumin in the sample was derived.

The PEG reagent and the diluent were allowed to reach RT. The antibody reagent was stored in aliquots of 1 ml at -20°C and diluted at 1/40 with PEG reagent on the day of the assay. The standard reference serum was stored at 4°C. On the day of analysis, the albumin concentration was checked by diluting the standard with diluent to give concentrations of 0, 10, 20, 50, 100 and 200 µg/ml. Quality control material consisted of a lavage sample from a normal volunteer (blood donor) to which serum from the same volunteer was added to simulate gut leakage as in IBD. This sample was stored in aliquots of 0.5 ml at -70°C and is stable for up to 1 year at this temperature.

A volume of 50 µl of diluent (B1), standards (SB) and test WGLF samples (TB), were each diluted with 0.95 ml PEG reagent in 2 ml polystyrene tubes, in duplicate, as blank values. The same volume of each corresponding sample (B2, S and T) were diluted with 0.95 ml of antibody reagent, in duplicate, in another set of 2 ml polystyrene tubes, as test samples. The WGLF samples were diluted with PEG reagent using the Gilson Dilugil, which was set to sample 0.05 ml and to dispense 0.95 ml. The turbidity of the blank and test samples were read after 15-20 minutes using a spectrophotometer set at the ABS mode at a wavelength of 340nm and a volume of 0.8. The PEG reagent was zeroed. Corresponding blanks and tests for each sample followed by PEG reagent were read.

Results were calculated by subtracting B2 from B1 (OD value for 0 ug/ml albumin), S from SB (OD value for 10 - 200 µg/ml albumin) and T from TB (OD values for test

samples). Using a Prism software program, a graph was plotted of OD values for standards. The test sample results were read from this graph, and the mean values were calculated and reported to the nearest whole number. Test sample results were only reported if the QC value was within $\pm 17\%$ of the expected value. The CV of this assay was 8.5%. The normal reference range, based on 62 volunteer subjects and patients with no known organic disease, was 0-26 $\mu\text{g/ml}$ (Brydon et al. 1993). Values greater than 26 $\mu\text{g/ml}$ were reported as indicating active IBD.

4.4 Immunohistochemical Methods

4.4.1 Common Reagents & Materials

1. **Cryospray 22** - Order code B1029. Bright Instrument Co Ltd., Cambridgeshire.
2. **Pap Pen** - Dako, Code 052002
3. **Slides** - Multispot x 100, PH 107, C A Hendley (Essex) Ltd., Loughton, Essex.
4. **Cover slips** - 22 x 50 mm, No 1.5 thickness, Life Sciences International (UK) Ltd., Hampshire.
5. **Embedding compound (OCT)** - Bright Cryo-M-Bed (113 mls), Bright Instrument Co Ltd.
6. **Acetone** - dried Analar grade product. BDH, Merck, Product code 1047
7. **Poly-L-lysine solution** - 0.1% w/v poly-L-Lysine in water (Sigma Product code P8920)
8. **Industrial Methylated Spirit (IMS99)** - obtained from our pharmacy stores
9. **70% IMS** = 700 ml IMS99 + 300 ml distilled water
10. **Hydrochloric acid Analar** - (Merck)
11. **Histoclear** - (BS&S Scotland Ltd)
12. **Lithium Carbonate** - (Merck)
13. **Xylene** - (Merck)

4.4.2 Instrumentation

1. **Bright Safecut 7000 Cryostat**
2. **Downflow workstation fume cupboard**
3. **Hund H500 Fluorescence microscope** - with appropriate filters for FITC fluorescence
4. **Dissecting Microscope**
5. **Leica Q500MC Image Analyser** - utilised a microscope, solid state colour video TV camera attached to a PC compatible control computer. An image from the microscope was transferred by the video camera to the monitor, and was then processed to give an object suitable for measuring. The object was converted into a binary image where different colour intensities were detected. The instrument can be used to identify parameters such as area, distance, number, shape, position and OD of identifiable parts of an image.

4.4.3 IgA Endomysium Antibodies

4.4.3.1 Specific Reagents

1. **Human Umbilical Cord (HUC)** - was obtained from the Simpson's Maternity Hospital, Edinburgh Royal Infirmary, as soon after delivery as possible. Sections of HUC were transversely cut into 5 mm tissue blocks, embedded in embedding compound and frozen rapidly using either cryospray or dry ice. The embedded tissue blocks were stored at -70°C. A sample of the cord blood was anonymously tested for Hepatitis B and HIV infection.
2. **Chloroform** - Analar grade, Merck Product Code 10077 6B, BDH
3. **Diluent** - 0.9% NaCl, 1% (v/v) adult bovine serum, 0.05% (v/v) Tween 20
4. **Conjugate** - FITC-conjugated sheep anti-human IgA (SAPU)
5. **Glycerol gelatin** - Sigma Product Code GG-1
6. **PBS tablets** - Sigma Product Code P4417

4.4.3.2 Procedure in Serum & Whole Gut Lavage Fluid

This method was developed and validated in our laboratory by Mr. John Bode and Dr. Helen Gillett. Cryostat sections of HUC (7 microns thick) were cut and transferred to multispot slides (3 sections per slide). Slides were wrapped in tin foil and stored at -20°C until use. Frozen sections were thawed at RT for 30 minutes and then fixed in acetone in a pre-cooled Coplin jar for 10 minutes at -20°C. Slides were then transferred under the downflow fume cupboard into a Coplin jar containing chloroform for 30 minutes at RT. Each section was covered with diluent for 10 minutes and then drained. Sections were then removed and allowed to dry at RT. A line was drawn between each section on the slide using a pap pen to create a wax barrier and prevent cross contamination. Diluent was added to each section in order to block non-specific binding, and left to stand for 5 minutes in damp staining box. The slides were then drained and not allowed to dry out before serum was added to each section.

Test serum samples were diluted 1/5 in diluent and WGLF were not diluted. 50 µl of each sample was added to individual sections and incubated at RT for 60 minutes. Positive and negative control sera were included in each batch. To determine the titre of EmA positive serum samples doubling dilutions from 1/25 to 1/200 were performed. Sections were rinsed in PBS and then 50 µl of conjugate (diluted at 1/20) was added to each section and the slides were incubated in a dark moist box for 60 minutes. A bottle of glycerol gelatin was placed onto a hotplate to liquefy while the slides were placed into a Coplin jar containing PBS and rinsed with running water for 5 minutes. Cover slips were warmed on the hotplate and glycerol gelatin was streaked onto cover slips. The slides were then dried and then mounted onto the cover slips.

Slides were examined by two trained independent readers (blinded to the patient details) at x10 and x25 magnifications using an immunofluorescence microscope. Reporting was qualitative with a positive result recorded if the connective tissue surrounding the

muscle cells fluoresced brightly in a honeycomb pattern. In serum, a positive result required a titre of $\geq 1/5$. An equivocal result was recorded if the immunofluorescent pattern was not definitely positive or negative, and did not simply represent disagreements between readers. Any sections on which there was disagreement were repeated in the next batch along with any equivocal samples. Stained slides were stored in the dark and fluorescence was visible several weeks after preparation with little deterioration of signal.

In 1995, one year after its introduction into routine laboratory practice, the technique was retrospectively audited. The EmA results of 99 patients whom had undergone small intestinal biopsy were compared (55 patients IgA EmA positive, 44 patients IgA EmA negative). Patients with SIgAD (n=2) and those with only raised IELs (n=17) were excluded. This yielded a sensitivity of 100% and specificity of 95%.

4.4.4 Anti-Reticulin Antibodies in Serum

This assay was performed with assistance from Mrs. Diane Anderson at the medical microbiology laboratory. These antibodies were measured for research purposes only to investigate if other CD-associated tissue antibodies were expressed in the IgA EmA negative patients. As the assay was qualitatively reported, a mixture of IgA EmA negative and positive sera were submitted for analysis with the microbiology technicians blinded to the IgA EmA result.

In brief, ARA antibodies were detected by an indirect immunofluorescent technique using a composite block of rat liver, kidney and stomach. Sections 5 μm thick were mounted onto glass slides and fixed in acetone for 5 minutes at RT. Test and positive control sera, diluted at 1/20 in PBS, were added to substrate sections and incubated at RT for 30 minutes. After washing in PBS, an IgA fluorescein-labelled anti-human globulin conjugate (Binding Site, Birmingham, UK) was added and incubated for 30

minutes. A mixed conjugate of IgG, A, and M antibodies (Atlantic antibodies, USA) was also used. After a further wash in PBS, slides were mounted in glycerol. A positive reticulin result was indicated by an apple green fluorescence at titres greater than 1/20. A report was issued for all samples submitted for analysis.

4.4.5 Haematoxylin & Eosin Staining

4.4.5.1 Specific Reagents

1. **1% Acid Alcohol** - 10 ml concentrated HCl acid + 990 ml 70% IMS
2. **Eosin Y water and alcohol soluble** - CI No 45380 (Difco)
3. **1% Eosin in 70% IMS** = 1 g Eosin + 100 ml 70% IMS
4. **Harris Haematoxylin** - Sigma Product code HHS-80
5. **DPX** - (Merk)

4.4.5.2 Procedure in Small Intestinal Biopsies

The H&E dye staining method is the routine stain of choice in most histopathology laboratories as it is suitable for tissue that has been fixed in most fixatives. Most tissue components are demonstrated and in most cases a diagnostic decision can be made without the need for further specialised staining procedure. All biopsies were submitted to the pathology laboratory for routine processing and reporting. A duplicate slide and histology report of each H&E stained small intestinal biopsy sample were sent to the GI laboratory.

In brief, the procedure required endoscopic duodenal biopsies to be mounted onto filter paper and fixed in formalin before transfer to the pathology laboratory. The biopsies were de-waxed in histoclear and then placed into IMS99, followed by rehydration in 70% IMS. Sections were then washed in running tap water, stained in haematoxylin and washed again. After this, sections were dipped 5 times in 1% acid alcohol and washed

again in tap water. Sections were then placed in saturated aqueous lithium carbonate until blue. Staining was checked using light microscopy to ensure that the nuclei were blue and the background was colourless. Repeated cycles of washing, dipping in 1% acid alcohol and saturating aqueous lithium carbonate were performed until the desired staining was achieved. Following this, sections were washed, counterstained in eosin for 5 minutes, and then washed again in running tap water. Sections were then placed sequentially in 70% IMS, IMS99, histoclear and xylene before coverslips, streaked with DPX, were applied and left to dry.

4.4.6 IEL Quantification Using Image Analysis

IELs are frequently raised in jejunal and duodenal biopsies of patients with untreated CD or DH. These cells can be demonstrated on H&E stained small intestinal biopsies. IELs were first quantified by counting epithelial cell nuclei in H&E stained small intestinal biopsies using light microscopy. For each biopsy, 500 epithelial cell nuclei were counted and the IEL count was expressed as cells per 100 epithelial cells (Ferguson and Murray, 1971). Using this technique, the normal reference range for IELs was between 6-40 IELs per 100 villous epithelial cells. As this technique was very time consuming, it was replaced by computerised image analysis. This method was developed and validated Dr. H. Gillett who examined 31 biopsies using both methods and found a positive correlation of 0.964 ($p < 0.001$) using linear regression. An upper reference limit of < 40 IELs/mm was calculated (Gillett and Ferguson, 1996).

IELs were quantified using H&E-stained sections of endoscopic duodenal biopsies under the x10 and x40 objectives. Like the previous method, this technique required well-orientated tissue sections and an intact surface epithelium. The computer was calibrated following each change of the objective and for the measurement of distance in millimetres. Using the computer's mouse, a line was drawn along the epithelial surface, as closely as possible, of a well-orientated area of tissue with an intact epithelial

surface. All IELs in the area of epithelium drawn along were selected by “clicking” on them in the counting mode of the program. The computer cumulatively added together the number of IELs for each section of biopsy measured until a distance of 1 mm was reached. The number of IELs was expressed per millimetre of epithelium (IEL/mm).

4.4.7 CD3 & γ/δ Staining & Quantification

4.4.7.1 Specific Reagents

1. **0.1% (w/v) poly-L-lysine** - Sigma code P 8920 - diluted 1/10 in distilled water
2. **Tris** - BDH analar, code 10315
3. **0.5M Tris buffer (pH 7.6)** - was prepared by dissolving 60.72 g of Tris in 500mls of distilled water adjusting the pH to 7.6 with 1N HCl.
4. **0.05M Tris buffered saline (TBS)** - was prepared by diluting 0.5M Tris buffer 1/10 in 0.9% sodium chloride (pH 7.6).
5. **Rabbit Serum** - SAPU, code S030-220 Batch No. 6208T
6. **Blocking Solution** - was prepared by adding 1 ml of normal rabbit serum to 4 ml of 0.05M TBS (NRS/TBS).
7. **Monoclonal Anti-CD3** - SAPU, Catalogue No. TA 1061
8. **Monoclonal Anti- γ/δ TCR** - (T Cell Diagnostics, Inc, Woburn, MA 01801) TCR 1061
9. **Biotinylated Rabbit Anti-Mouse Immunoglobulins** - Dako
10. **Streptavidin/biotin peroxidase (StrepABComplex)** - Kit from Dako, Code Lot
11. **Substrate Solution** - was prepared fresh in the downflow workstation by combining 10 mls 0.5M Tris pH 7.6, 5 mg Diaminobenzidine, 1 flake of Imidazole and 60 μ l 4 6% Hydrogen peroxide.
12. **Gill's number 1 haematoxylin** - Sigma code GHS-1-80
13. **Isopropanol**

4.4.7.2 Procedure in Small Intestinal Biopsies

The methods for the quantification of IELs expressing CD3+ and γ/δ -TCRs motifs were previously developed in this laboratory using an image analysis technique (Arranz et al. 1994). The principle of the method is that these cells can be detected by immunocytochemistry using monoclonal antibodies, anti-CD3 and anti- γ/δ , on cryostat sections and quantified using image analysis. Endoscopic duodenal biopsies were collected unfixed (samples processed in paraffin were unsuitable for staining) in a universal container and were mounted in a drop of OCT on a glass slide as soon after biopsy as possible. Each biopsy was orientated with the villous surface uppermost and the tissue lying flat using the dissecting microscope. The tissue was then rapidly frozen with cryospray, wrapped in tin foil, labelled and stored at -70°C until ready for staining.

Before tissue sectioning, slides were coated with 0.01% poly-l-lysine for 5 minutes and allowed to dry. Frozen biopsy tissue was transferred to the cryostat and bisected. Half of the specimen was embedded at right angles in OCT and then frozen onto a cryostat specimen holder. Serial sections 6 μm thick sections were cut at a temperature of -15°C to -20°C and placed onto the 0.01% poly-l-lysine coated slides and allowed to dry at RT for 30 minutes. The slides were then wrapped in tin foil and stored at -20°C . Unused tissue was covered in OCT, wrapped in tin foil and returned to the -70°C freezer. In preparation for staining, sections were removed from the freezer and allowed to rise to RT before removal of tin foil. The sections were thoroughly dried and were then fixed in acetone for 30 minutes at RT. After fixation, sections were allowed to dry at RT for 30 minutes and a PAP pen was used to draw around the sections to form a reservoir for the antibody solutions.

The staining technique was as follows. After being rehydrated in 0.05M TBS for 5 minutes and treated with blocking solution for 10 minutes, sections were drained but not allowed to dry. Monoclonal antibodies (diluted in blocking solution) were then applied

to the sections for 60 minutes. For identification of the CD3⁺ cells, anti-CD3 was added at a dilution of 1/20 and for identification of the γ/δ TCRs, a mouse anti- γ/δ TCR antibodies at a dilution of 1/80 was applied. After incubation and washing, a biotinylated rabbit anti-mouse IgG at a dilution of 1/400 in blocking solution was added. Following incubation and washing, the sections were treated with StrepABComplex in TBS and the reaction was visualised, under a downflow workstation, by adding the substrate solution. Each incubation was for 40 minutes and rinsing was with TBS for 5 minutes between applications. Sections were then washed in running tap water and counterstained with haematoxylin for 1 minute. Sections were then placed in 1% acid alcohol for 2 seconds, washed in running water and saturated in aqueous lithium carbonate until "blue". After a further wash, sections were sequentially placed in 70% IMS for 15 seconds, isopropanol for 20 seconds, histoclear for 5 minutes and xylene for 5 minutes, before coverslips were applied. Stained sections were stored in numerical order in cardboard slide boxes.

A known positive control section was stained together with the test sections and compared to its previous staining pattern and intensity. CD3 positive cells stained brown with the nuclei stained blue. Cell counts were quantified and the epithelial surface measured using the image analyser. Only positively stained cells in the epithelial cells of the villi were counted and the length of mucosa from the counted area measured. The computer was calibrated and measurements are carried out at x40 magnification. The results were expressed as the number of positive cells/mm of villous epithelium. Only those specimens in which at least 5 mm of intact epithelium could be counted were considered technically acceptable. The normal reference range was based on the analysis of 34 jejunal biopsies from patients who were considered to have an immunologically normal gut (Arranz et al. 1994). The upper reference limits (mean + 2 SDs) for CD3⁺ IELs and γ/δ TCRs were 67.5/mm and 5.5/mm of villous epithelium respectively in this group of subjects.

4.5 Morphometric Analyses

4.5.1 Micro-Dissection of Duodenal Biopsies

4.5.1.1 Specific Reagents & Materials

- 1. Clarke's Fixative**
- 2. 50% IMS** - 250 ml sterile water + 250 ml IMS99
- 3. Schiff's Reagent** – Sigma Cat. No. 395-2-016
- 4. 45% Acetic Acid**

4.5.1.2 Instrumentation

- 1. Water bath** – Grant Instruments Ltd., Cambridge
- 2. Thermometer**
- 3. Cataract knife** – Moria, Interfocus Ltd.

4.5.1.3 Procedure

This technique was used as a measure of small intestinal architecture and was adapted for use in human small intestinal biopsies by workers from this laboratory (Ferguson et al. 1977). Fresh endoscopic duodenal biopsies, mounted on filter paper, were placed into a glass container of Clarke's fixative for 24 hours. The fixative was carefully decanted from the container and replaced with IMS99 until ready for processing. The procedure was done in batches of 3-4 biopsies as it was found to be very time-consuming and conserved reagents.

On the first day of the procedure, the IMS99 was drained and the biopsies were sequentially placed into 70% IMS and 50% IMS, each for 15 minutes at RT. After this the 50% IMS was decanted and the biopsies were rinsed in sterile water 3 times for 5 minutes. The biopsies were then put into a jar containing 1M HCl for 10 minutes and placed into a water bath set at 60°C. Following this, the biopsies were rinsed again as

before in preparation for staining. The Schiff stain was placed into each container for 45 minutes and the biopsies were noted to turn purple in colour. The biopsies underwent another 3-rinse cycle and were stored in sterile water for no longer than 3 days. If longer storage was required, the biopsies were replaced into 50% IMS, but had to be rehydrated in sterile water for at least 15 minutes before proceeding.

On the second day of the procedure, the biopsies were dissected. Under light microscopy, the muscularis was removed from the tissue using fine forceps and the cataract knife, and an area with well-separated villi was selected. The biopsy was held at one end and single rows of villi were sectioned and placed onto a glass slide in 45% acetic acid. A cover slip was then gently applied, and the measurements of the villous height and crypt depth were made using the image analyser. At least 10 villi were measured from the base to the tip using the x10 objective on the microscope. The crypt depth was measured in at least 10 areas of the section using the x40 objective on the microscope. The mean of the 10 measurements was calculated. The normal reference limits for villous length and crypt depth were 500-1100 μm and 150-300 μm respectively (Ferguson et al. 1977). The number of mitoses/crypt was measured by gently, but firmly squashing the biopsy to liberate the crypts from the lamina propria matrix and to flatten them out. The number of mitotic figures in a range of crypts was counted. The normal reference range was 1-12. Morphometric measurements were difficult could not be made with formalin-fixed biopsy specimens, and were more difficult in small or traumatised biopsies.

4.6 Area & Adequacy of Small Intestinal Biopsies

4.6.1 Background

At the beginning of this research project, I conducted a retrospective audit of the adequacy of duodenal compared to jejunal biopsies submitted to the pathology department during the period from January to December 1996. This was undertaken to assess the policy change implemented in November 1996 from obtaining small intestinal biopsies using the Crosby capsule technique to the endoscopic method. The change to the endoscopic technique had the advantages of offering sedation, providing a more rapid examination and avoiding radiation exposure. This technique was also known to be preferred by patients (Achkar et al. 1986). It also allowed targeting of biopsies and immediate verification of the presence of tissue. This was important as the jejunal capsule biopsy technique has a failure rate of up to 20% (Mee et al. 1985; Achkar et al. 1986). Endoscopic mucosal appearances may also provide additional information by allowing direct visualisation and evaluation of the duodenal mucosa. Such features include the findings of a mosaic pattern in the duodenal mucosa after the application of indigocarmine (Stevens and McCarthy, 1976) and the reduction or absence of duodenal folds (Brocchi et al. 1988).

In our unit, a single specialist nurse performed all of the Crosby capsule biopsies with radiological guidance. Each biopsy was examined under the dissecting microscope and carefully orientated before fixation and transfer to the pathology laboratory. In contrast, the procedure for endoscopic duodenal biopsy had no set protocol and was performed by many different operators. The size of biopsy forceps used and number of biopsies taken were not standardised and there was no attempt made to orientate biopsies before fixation.

As the definitive histological diagnosis of CD relies on a sufficient amount of well-orientated tissue, this retrospective audit was undertaken to compare the area, adequacy and degree of damage of jejunal suction and endoscopic duodenal biopsies. The objectives were to determine if taking duodenal biopsies were an adequate replacement for obtaining jejunal biopsies, and to try to make recommendations to optimise the endoscopic biopsy technique. It was important to establish that endoscopic biopsies were adequate for diagnosis as histology was the “gold standard” for diagnosis by which the serological methods under investigation were going to be compared.

4.6.2 Methods & Statistical Analysis

The GI laboratory had retained H&E stained slides and duplicate pathology reports of all small intestinal biopsies performed in the unit for research purposes for many years. During the study period, there were 28 jejunal and 267 duodenal biopsies performed. All of the jejunal biopsies were selected. Duodenal biopsies (n=27) were randomly selected as every tenth biopsy after the fourth. The number of duodenal biopsies taken per patient was not routinely documented on the pathology reports and this was determined by the number of sections on each slide. The duodenal and jejunal biopsy cross-sectional areas, orientation and degree of damage were compared.

The cross-sectional biopsy area was measured in mm² with a computer program designed by Mr. John Bode using the Leica Q500MC image analyser. The number of duodenal biopsies taken per patient was recorded. As more than one duodenal biopsy was usually obtained per patient, the mean cross-sectional areas of the duodenal biopsies were compared with each single jejunal biopsy. The adequacy of each biopsy was independently assessed using light microscopy by two investigators, Dr. Alastair Lessels (AL), consultant pathologist, and myself (AD) blinded to the technique used to obtain the biopsy. The criteria used were based on previous reports (Achkar et al. 1986; Dandalides et al. 1989), as follows:

Excellent:	a minimum of 4 well orientated villi in a row
Good:	1-3 well orientated villi in a row
Poor:	no discrete villi or poor orientation of nuclei

The term "well orientated" was defined as consecutive villi projecting at right-angles to the surface mucosa (Dandalides et al. 1989). In cases of villous atrophy, an excellent biopsy required >2 crypts to be followed along their entire length running in parallel with the muscularis mucosae to the luminal surface. The orientation was considered to be good if only 1 or 2 crypts could be followed, and poor if crypts were cut tangentially (Dandalides et al. 1989). The degree of damage was subjectively assessed and graded as minimal, moderate or severe. Where multiple biopsies were taken, the best result for adequacy and damage were recorded.

The difference in cross-sectional areas between jejunal and duodenal biopsies were compared using the Mann-Whitney test. The Fisher's exact test and the Chi-squared (χ^2) test were used to determine if there was a statistical difference between the numbers of excellent jejunal and duodenal biopsies, and also to compare the degree of damage of jejunal and duodenal biopsies. Inter-observer variation was analysed using McNemar's test, which tests for a significant difference between disagreements.

4.6.3 Results

4.6.3.1 Cross-sectional Biopsy Area

A single jejunal biopsy had been obtained from each patient. The cross-sectional area of the jejunal biopsies ranged from 1.23 mm² to 5.54 mm² (median 2.78 mm², mean 2.9 mm²). The number of duodenal biopsies taken per patient ranged from 1-5 (median 2) biopsies. Each individual duodenal biopsies ranged in cross-sectional area from 0.28 mm² to 6.88 mm² (median 1.78 mm², mean 2.2 mm²). The mean cross-sectional area of

the duodenal biopsies taken per patient ranged from 0.36 mm² to 5.17 mm² (median 1.75 mm², mean 2.09 mm²). The jejunal biopsies were significantly larger than the individual duodenal biopsies (Mann-Whitney test: $p=0.01$) and the mean duodenal biopsy area per patient (Mann-Whitney test: $p=0.02$) (**Figure 4.1**).

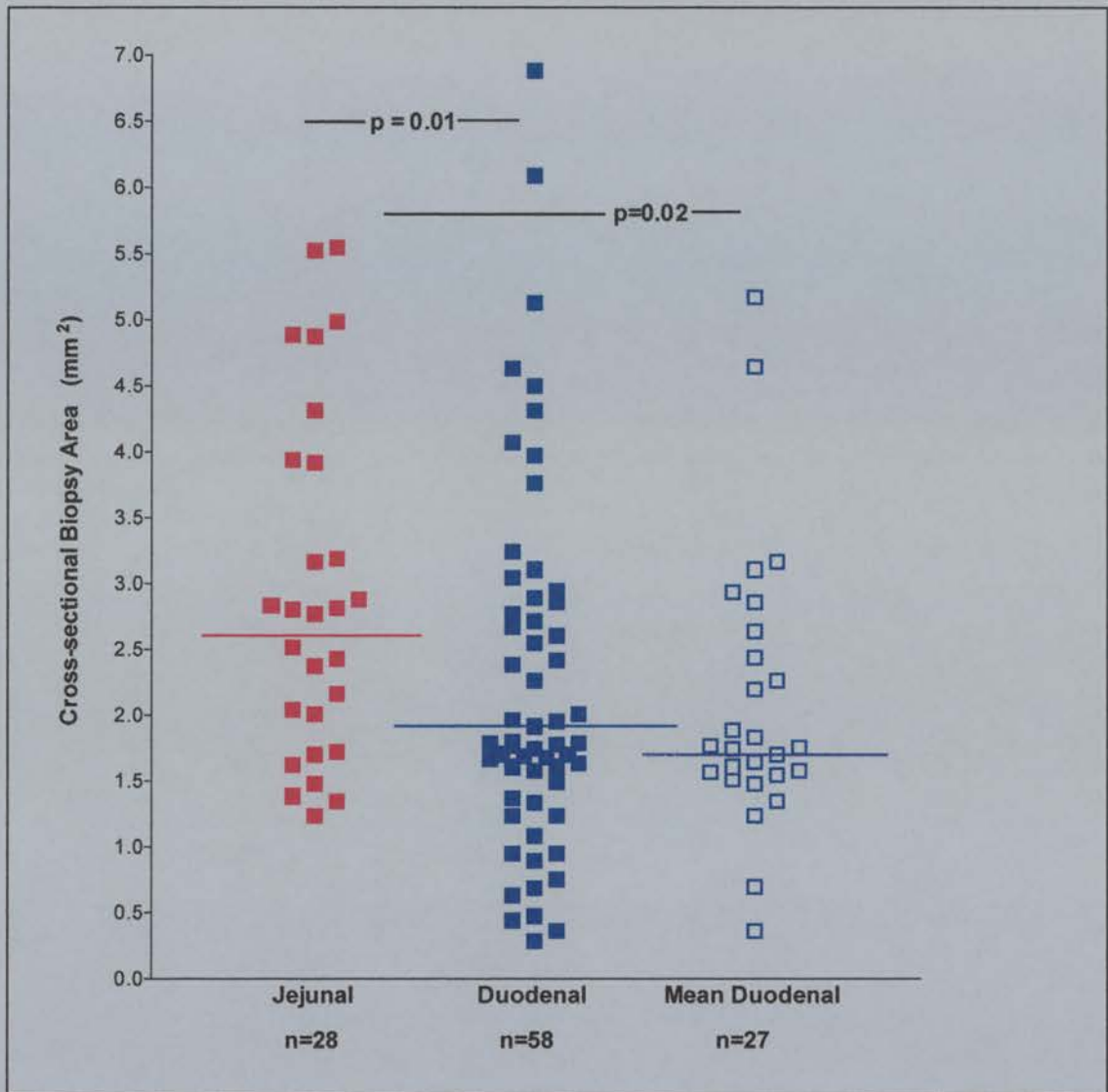


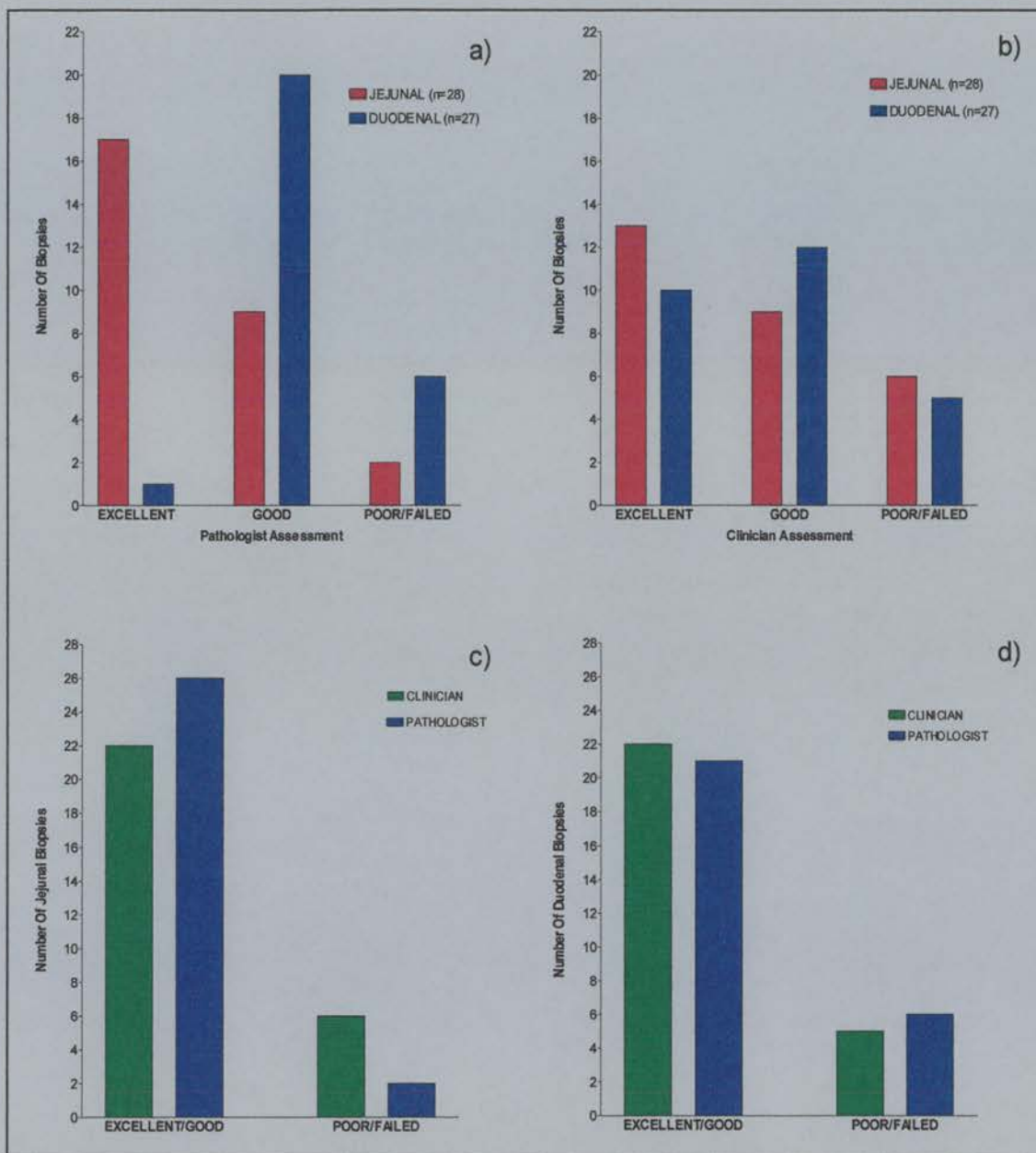
Figure 4.1: Comparison of the cross-sectional areas of jejunal & duodenal biopsies. The cross-sectional areas of the jejunal biopsies (n=28), as well as each individual duodenal biopsy obtained (n=58) and of the mean cross-sectional area of duodenal biopsies obtained per patient are shown. The median cross-sectional area of the jejunal biopsies, indicated by the horizontal line, was significantly larger than that of the duodenal biopsies.

4.6.3.2 Assessment of Adequacy

The Crosby capsule failed to obtain a jejunal biopsy in 1/28 (3.6%) patients. In this patient, the capsule was fired too proximally and gastric mucosa was obtained. Using the endoscopic technique, insufficient duodenal tissue was taken from 2/27 (7.4%) patients and in another patient, a duodenal ulcer was biopsied. In this subject, only 1 biopsy had been taken.

AL found that jejunal biopsies had significantly more excellent results 17/28 (60.7%) than duodenal biopsies 1/27 (3.7%) (Fisher's exact test: $p=0.039$) (**Figure 4.2a**). AD did not find any significant difference in the number of excellent results in jejunal: 13/28 (46%) compared to duodenal biopsies: 10/27 (37%) (χ^2 test: $p=0.48$) (**Figure 4.2b**). A confident histological interpretation could be made if biopsies were graded as excellent or good, and therefore the analysis was repeated when the number of excellent and good biopsies were combined. This showed that there was no significant difference in the adequacy of jejunal and duodenal biopsies (**Figures 4.2 c-d**).

Villous atrophy was present in 3/28 (11%) of jejunal and 2/27 (7%) of duodenal biopsies. AL and AD both agreed that all 3 jejunal biopsies were excellent. AL graded one of the 2 duodenal biopsies as good and the other poor. AD found that both the duodenal biopsies were good.



Figures 4.2 a-d: Comparison of the adequacy of jejunal & duodenal biopsies. The pathologist found jejunal to be superior to duodenal biopsies, but both observers found jejunal and duodenal biopsies to be adequate when excellent and good biopsies were combined.

4.6.3.3 Assessment of Damage

AL found that jejunal biopsies were significantly less damaged than the duodenal biopsies (χ^2 test: $p=0.005$) (**Figure 4.3a**). AD found no significant difference in the degree of damage of the jejunal and duodenal biopsies (χ^2 test: $p=0.91$) (**Figure 4.3b**). Both AL and AD agreed that the 3 jejunal and 2 duodenal biopsies with villous atrophy were minimally damaged. A histological diagnosis could not be made in 1/28 (3.6%) of jejunal and 2/27 (7.4%) of duodenal biopsies due to inappropriate tissue submitted or to severely traumatised specimens.

4.6.3.4 Assessment of Inter-Observer Variation

Comparison of the independent reporting by AL and AD was made using McNemar's test. This showed that there was significant inter-observer variation in the assessment of adequacy ($0.01 > p > 0.001$) and damage ($0.05 > p > 0.02$). There were however, no instances in the reporting of adequacy where the disagreement was by more than one grade, and there was only one occasion in assessing the degree of damage where the disagreement was by more than one category. This occurred in the assessment of a jejunal biopsy in which AD found it to be severely damaged and AL only minimally.

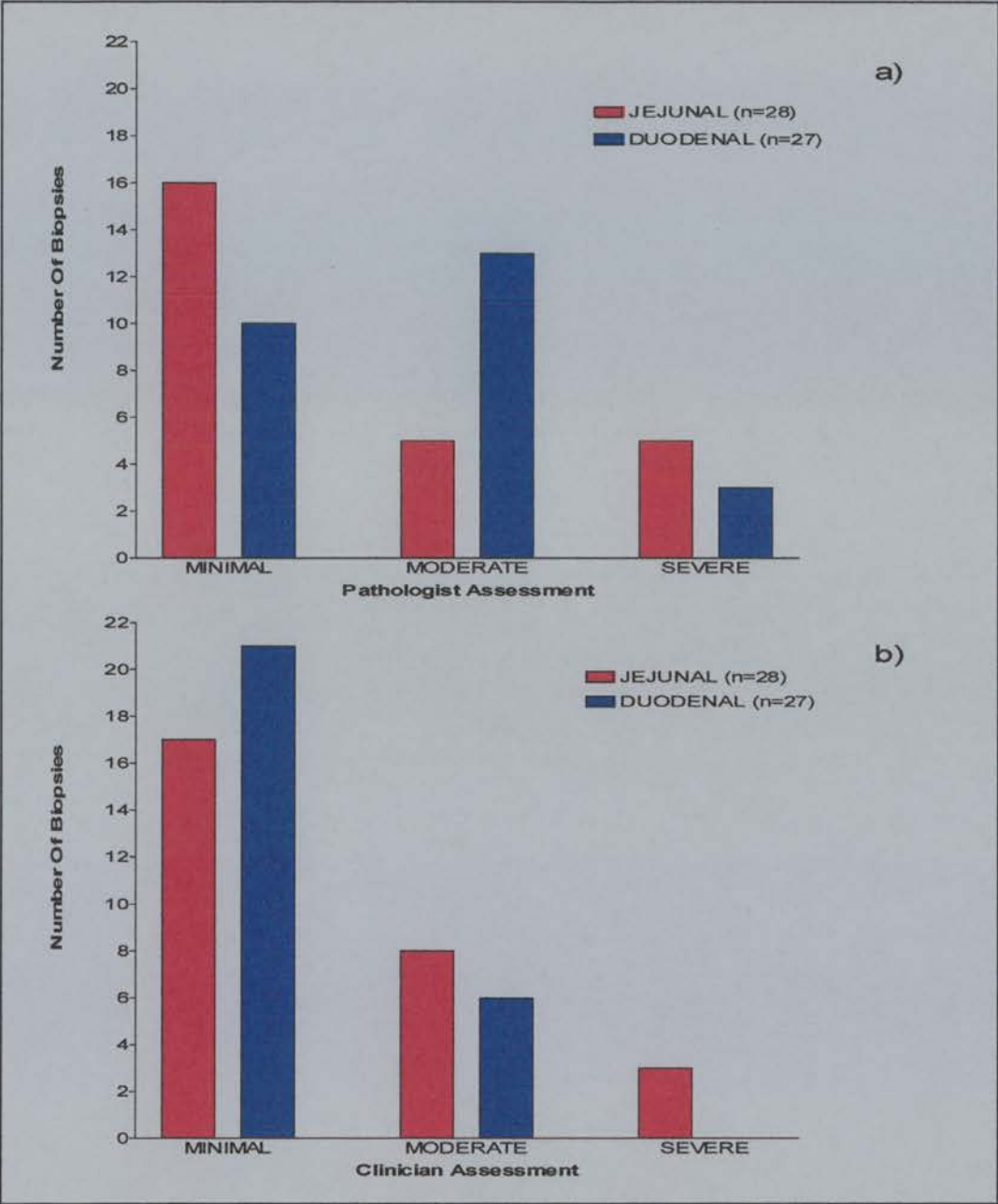


Figure 4.3a & b: Comparison of the assessment of damage of jejunal & duodenal biopsies

4.6.4 Discussion

This audit showed that although jejunal biopsies are larger than duodenal biopsies, the latter are adequate for the diagnosis of CD when the number of excellent and good biopsies were combined. The smaller duodenal biopsy size is known to be due to the limited size of the forceps permitted to pass through the biopsy channel of the endoscope (Shidrawi et al. 1994). Others have shown that although jejunal biopsies were significantly larger than endoscopic biopsies, the latter were adequate for making the diagnosis of CD provided that four biopsies were taken (Mee et al. 1985).

The pathologist found that jejunal biopsies were less damaged than duodenal biopsies, however I did not find any significant difference. We agreed on the assessments of adequacy and damage for all of the jejunal and duodenal biopsies in which there was evidence of villous atrophy. Duodenal biopsies tend to be more prone to trauma because they may be crushed within the biopsy forceps and as they are small, they may be damaged by the needle when they are being transferred from the biopsy forceps to the filter paper. A change in policy with more care during mounting of biopsies or direct transfer into formalin without mounting may reduce the degree of damage to biopsies.

There was significant inter-observer variation in reporting despite agreed criteria for adequacy. This was due to a difference in the interpretation of a well-orientated villous. The assessment of the degree of damage was more subjective than that for adequacy. A histological diagnosis could be made in all but 3.6% of jejunal biopsies and in 7.4% of duodenal biopsies because of inappropriate site biopsied and severely damaged mucosa. A recent study reported that 87.5% of endoscopic biopsies and 94.2% of capsule biopsies were adequate for histological diagnosis (Branski et al. 1998). They found that fragmentation or squashing was seen in 83.3% and 25% of endoscopic and capsule biopsies respectively. An audit has compared the incidence of villous atrophy in the 5 years after changing to endoscopic duodenal biopsy with the preceding 9-year

experience of jejunal capsule biopsy (Saverymuttu et al. 1991). It showed that the annual new case detection rate of CD rose significantly from a mean of 1.9 to 4.6 ($p<0.05$) after the change to endoscopic biopsy. This was in part due to three-fold increase in the number of subjects investigated by small intestinal biopsy.

In this audit, there was one instance where a duodenal biopsy was too inadequate to allow reporting, and only one biopsy had been taken. As the mucosal changes in CD may also be patchy (Scott and Losowsky, 1976), taking multiple biopsies may reduce the chance that these abnormalities would be missed. To my knowledge, there are no reports of the adequacy of duodenal biopsies if varying numbers of biopsies were taken. Most investigators have reported that a minimum of 3-4 biopsies should be taken (Shidrawi et al. 1994; Mee et al. 1985; Branski et al. 1998). Dr. Lessels advised that 4 endoscopic biopsies should provide sufficient material to make a diagnosis. This would avoid the need to attempt to orientate these specimens before fixation.

This audit highlighted the lack of documentation and standardisation of endoscopic biopsy forceps size and the number of biopsies taken. Since completing this audit, I have routinely used large biopsy forceps and obtained 3-4 endoscopic biopsies from all patients with suspected CD. I found that the procedure was well tolerated. Although the audit showed that our endoscopic biopsy policy in 1996 allowed a diagnosis to be made in the majority of patients, there was still room for improvement. I have not prospectively studied the adequacy of endoscopic biopsies after implementing these changes, as duodenal and jejunal biopsies gave comparable excellent and good results. Standardisation of the forceps size and number of duodenal biopsies taken should help to produce more excellent results. The endoscopic approach has provided a more ready access to the upper small bowel, and duodenal biopsies are a satisfactory replacement for suction biopsy in the routine investigation of patients with suspected CD.

4.7 HLA-DQ2 Analysis of Coeliac Disease Patients

The HLA-DQ2 typing was performed by Kathleen Kingstone and the technique was discussed in full in her thesis (Kingstone, 1997). In brief, an EDTA (full blood count) blood sample was obtained from each prospectively diagnosed CD patient for HLA-DQ2 analysis. DNA analysis for HLA-DQ α 1*0501 + DQ β 1*0201 (DQ2) were determined in DNA extracted from leukocytes. Polymerase chain reactions (PCR) were carried out using appropriate primers (Bell et al. 1989; Larhammar et al. 1983) to amplify the relevant regions of chromosome 6. PCR products were dot blotted onto nylon membrane and hybridised with digoxigenin labelled probes. The presence of hybridised products was determined using fluorescent detection. Boehringer Mannheim Biochemica kits Product Numbers 1175033 and 1363514 were used to label the appropriate probes and detect the hybridisation respectively. Positive and negative control sera were used in each batch.

4.8 Lactulose/Rhamnose Sugar Permeability Test

4.8.1 Specific Reagents

1. **Lactulose** - 5 g (4-O- β -D-galactopyranosyl-D- fructofuranose) (Sigma Cat. No. L7866)
2. **α -L Rhamnose** - 5 g (6-deoxy-L-mannose) (Sigma Cat. No. R3875)
3. **Sugar Solution** - 5 g lactulose, 1g rhamnose, 20 g sucrose and 20 g lactose (final osmolarity 1500mosmols) was dissolved in 80 mls boiling water, which is allowed to cool.
4. **Sodium Thiomerol Solution** - (Sigma Cat No. T-5125) dissolved in distilled water at 10 mg/ml and was stored at 4°C.
5. **Water** - high performance liquid chromatography (HPLC) grade (Cat. No. 1020 4 x 2.5 L, Rathburn Chemicals Ltd., Scotland)

6. **EDTA: Disodium Calcium Salt** (Ca/Na₂ EDTA) - (Sigma Cat. No. ED2SC, 100g)
7. **HPLC Solvent** - Dissolve 0.125 g Ca/Na₂ EDTA in 2.5 L HPLC grade water, filter under vacuum through 0.45µm filter and store at 4°C.
8. **Raffinose Hydrate** - 25 g BDH (Merck Cat. No. 38056)
9. **Stock Standard Solution** - 10 g/l in 0.9% NaCl (10 g/l lactulose + 10g/l rhamnose)
10. **Working standard solution** - 1mg/ml. Stock standard solution was diluted to 1mg/ml in 0.9% NaCl. 100 ml was made up and stored in 3.5 ml aliquots in sample tubes at -20°C.
11. **Standard samples** - standards diluted in NaCl at 0.1, 0.5 and 1.0 mg/ml were analysed.
12. **Internal standard solution** - 5 mg/ml in distilled water and stored at 4°C.

4.8.2 Instrumentation

1. **Waters™ Vacuum Manifold** - Waters Sep-Pak® Vac/3cc Alumina A cartridge - Part No. 20820 Millipore (UK) Ltd., Waters Chromatography Division, Hertfordshire.
2. **Waters HPLC system** - comprising the following modules:
 - Pump - Model 501 incorporating a 20 µl Rheodyne injection valve
 - Column heater
 - Differential refractometer - Model R401
 - Data module - Model 740
3. **Waters Sugar Pak™ column** - 6.5 x 300 mm Part No. 85188. Milipore (UK) Ltd. as above.
4. **Carbo-C precolumn** - Cat No. 125-0128 Biorad Laboratories Ltd., Hertfordshire
5. **Hamilton syringe** - 250 µl
6. **Heated magnetic stirrer**

4.8.3 Procedure

The sugar absorption test was used to demonstrate if malabsorption was of small bowel origin. The method was developed and validated in our laboratory (McDonald et al. 1991), and all tests were analysed as part of the routine laboratory service. The test was performed as an out-patient in most cases. The patient fasted from 22:00 hours on the day before the test, avoiding fruit and alcoholic beverages during the evening. On the morning of the test, the patient voided and discarded urine before starting the sugar test. The patient drank the sugar solution and was then not permitted to take any food or drink for the next 2 hours. After this period, the patient was allowed to take a minimum of 500 mls water to encourage a diuresis but has nothing to eat until the end of the urine collection. All urine passed in the 5-hour period after drinking the sugar solution was collected, and the patient was encouraged to empty his/her bladder at the end of this time. The 5-hour urine volume was determined by weight and a 10ml aliquot was retained for analysis in a plain sample tube. Any 5-hour urine collections that were less than 200ml were diluted 1/2 with water. 100 µl of sodium thiomersal solution was added to prevent bacterial breakdown of the sugars and the sample was stable stored at -20°C for up to 1 year.

Measurement of the intact sugars in the 5-hour collection of urine used an HPLC method with internal standardisation. Calculation of the ratio between the sugars emphasised any changes in epithelial integrity, and eliminated patient differences in gut transit time and renal function. Reference standard solutions (stock, working and internal) were prepared as above. Standard samples of 0.1, 0.5 and 1.0 mg/ml were made up. The QC sample was a fasting urine sample from a hepatitis B and HIV negative volunteer that refrained from eating fruit or taking alcoholic beverages for 24 hours before the collection. The urine was tested for its suitability by ensuring that there were no peaks that would interfere with sugar peaks, and then spiked with lactulose and

rhamnose to give a result on the upper limit of the reference range. The QC material was stored in aliquots at -20°C.

QC aliquots and test urine samples were thawed and centrifuged at 1500g for 10 minutes before analysis. A 2 ml aliquot of each was pipetted into polystyrene tubes. 200 µl of internal standard was added to each standard, QC and sample tube and then mixed. One Sep-Pak cartridge was activated per standard/QC/sample by running 5 mls of distilled water through the cartridge. Following this, 1 ml of standard, QC or sample was run through the cartridge to waste then the 2nd ml was added to the cartridge and the eluate was collected in polystyrene tubes in the vacuum manifold. The samples were then capped and were ready for injection. Up to 10 samples could be processed at a time.

The sugars were quantified using a Waters HPLC technique. Sugars were separated on a Sugar-Pak column maintained at 90°C and detected by differential refractometry. Test results were calculated from the standards with internal standard correction. Results were expressed as a ratio of % lactulose dose to % rhamnose dose excreted in 5 hours (L/R). The reference range for L/R ratio of 0.008 to 0.040 was calculated from 20 normal volunteers and confirmed from 600 patients using a probability plot (Kathleen Kingstone personal communication). The lower limit of detection was 0.01 mg/ml and the between batch CV was 8%.

4.9 Statistical Methods

The performance of the diagnostic tests used was described in terms of the sensitivity and specificity in diagnosing CD. The sensitivity was defined as the proportion of CD patients who were correctly identified by the test, while the specificity described the proportion of non-CD patients who were correctly identified by the test (Jones and

Payne, 1997b). The predictive power of the diagnostic tests was calculated to give an indication of the value of the test in predicting the probability that a patient did or did not have CD by transforming the sensitivity and specificity data. These were expressed as the PPV and NPV respectively.

Ideally a diagnostic test should have a 100% sensitivity and specificity, but this seldom occurs as there is usually some overlap between disease and healthy populations. This leads to the finding of “false positive” and “false negative” results. The strategy for choosing a suitable “cut-off” point differentiating positive and negative results depends on the perceived clinical cost of inaccurate diagnosis (Jones and Payne, 1997b). In CD, it would be desirable for serological methods to have a high sensitivity to limit the number of patients missed by the test, but this may be at the cost of a reduced specificity leading to more patients undergoing unnecessary investigations.

In this study the optimum cut-off concentrations for IgA anti-tTG antibodies in serum and WGLF, as well as WGLF IgA AGA, were determined using a non-parametric method at the 95th percentile (Jones and Payne, 1997b). The optimum cut-off point was also studied using receiver operating characteristic (ROC) curves. This graphical approach plotted the sensitivity against 1- specificity for each possible cut-off and the points were joined (Altman, 1991b). In a test with a high specificity, the curve follows the y-axis. The best cut-off point that balanced the “cost” of a false negative or false positive result, was that which maximised the sensitivity and specificity. This point was the point nearest the top left-hand corner of the graph. This method was used to compare the performance of the IgA anti-tTG ELISA in serum and WGLF across a wide range of antibody concentrations.

The SD and CV were used as a measure of the imprecision of the serological methods. The SD gives a measure of the variability of individual values from the mean, which

was the sum of all of the observations divided by the number of observations (Altman, 1991c). The CV should be determined at values that are “high”, “medium” and “low” in terms of clinically encountered results (Jones and Payne, 1997a).

The Mann-Whitney test, a non-parametric method, was applied to compare data from two independent groups. The test required all observations to be ranked as if they were from a single sample and then the sum of the ranks in the two groups were compared to test the null hypothesis that they were samples from the same population (Altman, 1991a). This method was applied to compare the concentrations of IgA anti-tTG antibodies, IgA AGA and IgG AGA in the serum and WGLF of CD patients and control subjects. This test was performed using the Minitab for Windows program (1994 version). The median was defined as the value that was found halfway in ranked data. The range stated the lowest and highest of the observed results. These analyses were performed using the Prism program for windows (Graph Pad Software Inc., 1994).

The χ^2 test was applied using two by two tables of independently classified data to test the null hypothesis that there was no relationship between rows and columns (Jones and Payne, 1997a). This test was performed using the Minitab for Windows program (1994 version). If the total frequency of observations in the two by two table was less than 20, or if the total lay between 20 and 40 and the smallest expected value was less than 5, then the χ^2 test with Yates correction was applied. To obtain the significance value (p), the χ^2 value was then compared with the p values at 1 degree of freedom in a standard χ^2 distribution table (Swinscow, 1983). The Yates correction was applied to compare the frequency of CD patients with a positive serum or WGLF IgA EmA in DQ2 positive and negative CD patients, the frequency of raised serum or WGLF IgA anti-tTG antibody concentrations in CD patients with varying degrees of mucosal atrophy (Marsh grade), and the frequency of a positive IgA EmA in patients with a raised IEL count.

Pearson's correlation was applied to determine the presence of a significant relationship between two variables. This was used to determine the correlation between serum and WGLF IgA anti-tTG antibody concentration with the IgA AGA concentration, IEL, CD3 and δ/γ T cell counts, morphometric measurements and the L/R ratio. It was also used to compare the relationship between the serum IgA anti-tTG antibody concentration and the corresponding IgA EmA titre, and that between the WGLF IgA anti-tTG antibody concentration and the WGLF total IgA. The significance was determined by linear regression using the Prism program for windows (Graph Pad Software Inc., 1994).

The Fisher's exact test was applied to compare the adequacy and degree of damage of jejunal and duodenal biopsies. This test was applied as the frequency of observations in the four-fold tables were too small for the χ^2 test. The McNemar's test was applied to test a significant difference in the inter-observer variation in determining the adequacy and degree of damage in jejunal and duodenal biopsies. This test was used as it enabled the comparison of the frequency of qualitative results recorded as one of 3 alternatives (excellent, good and poor for adequacy) or (minimal, moderate or severe for damage). The latter two methods were applied following the advice from Dr. Bill Adams, Statistician at the University Of Edinburgh Medical School, using an SPSS computer program.

CHAPTER 5: SERUM IgA ANTI-TISSUE TRANSGLUTAMINASE ANTIBODIES IN COELIAC DISEASE & OTHER GASTROINTESTINAL DISORDERS

5.1 Introduction

Serological testing has become an important non-invasive tool for screening patients presenting with symptoms or signs of malabsorption, and patients with conditions known to be associated with CD. Serology is also commonly used for monitoring gluten withdrawal and in assessing dietary compliance. The reported sensitivities and specificities of the available assays vary widely due to a lack of standardised methodologies, diagnostic practices and the varying populations studied. The sensitivities and specificities of the IgA EmA, IgA AGA and IgG AGA assays have varied from 74-100% and 96-100% (Hallstrom, 1989; Volta et al. 1991; Kolho and Savilahti, 1997; Valdimarsson et al. 1996; Cataldo et al. 1995), 46-100% and 86-100% (Unsworth et al. 1983; Bode and Gudmand-Hoyer, 1994; Volta et al. 1986; Volta et al. 1983), and 62-100% and 79-97% (Carroccio et al. 1996; Bode et al. 1993; Bode and Gudmand-Hoyer, 1994; Volta et al. 1983) respectively (**see Table 2.2**).

The finding that tTG is the autoantigen which interacts with EmA antibodies in CD sera (Dieterich et al. 1997), has allowed the development of a semi-quantitative ELISA. The measurement of IgA anti-tTG antibody concentrations in the serum of untreated CD patients has the potential advantage of providing an objective result as opposed to the subjective immunofluorescent IgA EmA result. The reported sensitivity and specificity of the IgA anti-tTG antibody ELISA have ranged from 85-98% and 90-98% respectively

(Sulkanen et al. 1998b; Dieterich et al. 1998; Lock et al. 1999b; Biagi et al. 1999a; Troncone et al. 1999). The majority of the untreated CD patients in these studies however had positive IgA EmA results.

This study differs from other recent reports by evaluating the IgA anti-tTG antibody ELISA in a large group of unselected untreated CD patients, as well as disease and healthy controls. We have also compared the sensitivity of the IgA anti-tTG antibody assay with the conventionally used IgA EmA, IgA AGA and IgG AGA assays. As tTG is widely distributed in the gut, we have also investigated the disease specificity by analysing sera from patients with a wide range of GI disorders, including inflammatory, infectious, functional and neoplastic aetiologies.

5.2 Patients

All patients recruited in this study had presented to the GI unit at the Western General Hospital in Edinburgh (**Table 5.1**). The patient demographics and clinical details, serum sample numbers, sample dates, serology and biopsy results were all entered into a Microsoft Access database to allow easy reference to and interpretation of results.

Untreated CD Patients:

A total of 102 untreated CD patients (72 F: 30 M; age range 15-78 years - median 47 years) diagnosed between 1988 and 1999 were studied. Of these 102 patients, 49 patients were prospectively diagnosed during the study period (October 1997 and April 1999). Small bowel mucosal biopsies were performed where clinically indicated and not exclusively following the finding of positive serological tests. All of these patients had histological evidence of CD on small intestinal biopsy and responded clinically to gluten withdrawal. The small mucosal histology in the 102 untreated CD patients

studied showed sub-total villous atrophy (STVA) in 46 patients, partial villous atrophy (PVA) in 50 patients and raised IEL counts in 6 patients.

All of these patients were on a normal gluten-containing diet at the time of serum sampling. These patients had been referred to the GI unit for investigation of abdominal symptoms, anaemia, weight loss or because of the presence of an associated disorder: IDDM (n=2) and collagenous colitis (n=1). Approximately 30 ml of blood was obtained and stored at -70°C for serological analysis. Venous blood was also taken for routine measurement of biochemical and haematological parameters as previously described in **section 4.1.1**. Serum samples were later obtained from 32 of these CD patients following gluten withdrawal (range 2-12 months - median 5 months) for repeat serological testing. In addition, serum samples stored at -70°C were available for 53 biopsy-proven, untreated CD patients diagnosed between 1988 and 1996. The prospectively diagnosed IgA EmA negative CD patients were offered a repeat biopsy following treatment with a GFD to support the diagnosis of CD.

DH Patients:

A total of 25 DH patients on a normal gluten-containing diet (6 F: 19 M; age range 19-76 years - median 52 years) diagnosed between 1989 and 1999 were studied. Most of these patients had been referred to the GI unit by the Edinburgh Royal Infirmary Dermatology department to investigate if these individuals also had CD. As the dermatology department had previously co-operated in recruiting patients for CD research, a written request was made to encourage referrals. Despite this however, only 6 of the 25 patients (1 F: 5 M) studied were referred during the study period. All of the 25 patients studied had histological confirmation of granular subepidermal deposits of IgA on skin biopsies, but only 21 patients had histological evidence on small intestinal biopsy of concomitant CD. This small intestinal histology in these 21 subjects showed total villous atrophy (TVA) in 2 patients, STVA in 8 patients, PVA in 9 patients, and raised IELs in 2 patients. Only 4 of the 6 prospectively diagnosed DH patients had

histological evidence of CD. Serum samples stored at -20°C were available for all of these patients.

Treated CD Patients:

Serum samples from 82 treated CD patients (62 F: 20 M; age range 15-82 years, median 53 years) who were established on a GFD for more than one year were analysed. These patients had all attended the GI unit coeliac clinic between 1998 and 1999 where serum had routinely been withdrawn for the measurement of IgA EmA and IgA AGA to monitor dietary compliance. Only patients with a negative IgA EmA and a dietary history of compliance were included. Small intestinal biopsies were not repeated in these treated CD patients at the time of this study.

Non-CD Controls:

Serum samples from 65 patients (46 F: 19 M; age range 16-90 years, median 45 years) who underwent small intestinal biopsies for suspected CD were included. Small intestinal histology was reported as normal in all of these patients. The identity of these patients was determined by obtaining an alphabetical list of all of the IgA EmA negative serum samples submitted from Western General Hospital patients between the middle of 1996 to the end of 1998 from the main laboratory computer Microsoft Access database. The total numbers of IgA EmA negative serum samples submitted each year were 209 for 1996, 529 for 1997 and 705 for 1998. Some of these samples were obtained from the same patient on more than one hospital visit.

To identify the patients who had a small intestinal biopsy obtained, the patient details corresponding to each serum sample was compared with the small intestinal pathology reports. Copies of small intestinal histology reports had been retained in the GI laboratory for many years. As these reports were in order of histology number, it was helpful to have the patients' surnames in alphabetical order to hasten cross-referencing. All of the treated CD patients with a negative IgA EmA and normal histology were

excluded. Of all the non-CD patients identified, only 102 patients had a negative IgA EmA and normal small intestinal histology. Stored serum samples (at -20°C) however, were available for only 65 of these patients.

Disease Controls:

Disease control serum samples were obtained by making a list of all of the patients who underwent a WGL during the year of 1996. The main laboratory computer Microsoft Access database started at the July of 1996. Before this, all samples submitted to the GI laboratory for analysis were manually recorded in a diary. During 1996, there were a total of 356 WGL procedures performed for which serum and WGLF samples were obtained. Another Microsoft Access database was created to store the patient demographics, sample details, and serum and WGLF results. Of the 356 WGL procedures performed, only 259 serum samples (from 245 patients) were still available. All serum samples obtained had been stored at -20°C and had not previously been used.

The diagnoses of these patients were confirmed by referencing the hospital casenotes and computerised records. The diagnoses of these patients were ulcerative colitis (UC, n=80), Crohn's disease (n=68), irritable bowel syndrome (IBS, n=36), infective or idiopathic diarrhoea (n=15) and miscellaneous other diagnoses including GI malignancy and liver diseases (n=46). Small bowel mucosal biopsies were not available for all of these patients.

Healthy Volunteers:

Serum samples from 29 healthy volunteers were prospectively obtained for analysis. These included 20 members of laboratory and secretarial staff, and 9 volunteers participating in an *E. Coli* research project. All samples were stored at -20°C and had not previously been used.

Table 5.1: Disease Categories & Patient Demographics

Disease Category	Number of Samples	Number of Patients	Gender (F: M)	Age Range (years)	Median Age (years)
<i>Untreated CD</i>	<i>102</i>	<i>102</i>	<i>72:30</i>	<i>15-78</i>	<i>47</i>
DH	25	25	6:19	19-76	52
Treated CD	82	82	62:20	15-82	53
Non-CD Controls	65	65	46 :19	16-90	45
UC	82	80	34:48	15-80	46
Crohn's Disease	80	68	46: 34	17-82	41
IBS	36	36	25:11	16-91	39
Infective/Idiopathic Diarrhoea	15	15	9:6	22-78	51
Other Diagnoses	46	46	28:18	19-82	54
Healthy Volunteers	29	29	19:10	21-58	35
TOTAL	562	548			

Number of disease control samples = 259

Number of disease control patients = 245

5.3 Methods & Statistical Analysis

The procedures for serum sample collection and processing have been previously described in **section 4.1.1**. All serum samples were analysed for the presence of IgA anti-tTG antibodies, IgA AGA and IgA EmA using methods described in **sections 3.3, 4.2.3, and 4.4.3** respectively. Serum IgG AGA was measured in all of the untreated CD patients, DH patients and non-CD controls (but not in the treated CD patients, disease controls or healthy volunteers), using the method described in **section 4.2.4**. Serum IgA EmA antibodies titres were measured in 37 of the untreated CD patients by performing doubling dilutions of the serum samples until the positive immunofluorescent pattern was lost. Serum total IgA was measured in all untreated CD patients as part of the routine clinical chemistry laboratory service (reference range 1.01-4.23 g/l). The upper reference limits of serum IgA anti-tTG antibodies, IgA AGA and IgG AGA were ≥ 2950 units/ml, ≥ 30 units/ml and ≥ 45 units/ml respectively. The method used for obtaining the upper reference limit of serum IgA anti-tTG antibody concentration was previously described in **section 3.1.6**. IgG anti-tTG antibodies were not measured due to the poor reproducibility of the assay as discussed in **section 3.4.7**.

The statistical methods applied were described in **section 4.9**. The sensitivity of the IgA anti-tTG antibody ELISA was calculated as the frequency of positive results in the biopsy-proven CD patients while on a normal diet, excluding IgA deficient patients. The specificity of the assay was calculated from the group of non-CD controls found to have normal duodenal histology and was not based on the disease controls from whom intestinal biopsies were not available for all patients. The Mann-Whitney test was used to compare IgA anti-tTG antibody concentrations in untreated CD and DH patients, with the control groups. Pearson's correlation was applied to determine the correlation between serum IgA anti-tTG and the corresponding IgA EmA titre, and the significance was determined by linear regression.

5.4 Results

5.4.1 Serum Total IgA in Untreated Coeliac Disease Patients

Of the 102 untreated CD patients only 2 patients had SIgAD (total serum IgA < 0.05 µg/l). One patient had STVA and the other PVA on initial small intestinal biopsies. Repeat duodenal biopsy histology had markedly improved in both of these patients following treatment with a GFD. Serum IgA-class anti-tTG antibodies, EmA and AGA were not detectable in these patients, but the IgG AGA concentration was raised. IgA-class serology results from these 2 male patients have been excluded from the calculations of assay sensitivity (n=100).

5.4.2 Serological Results in Untreated Coeliac Disease Patients

Serum IgA anti-tTG antibody, IgA AGA and IgG AGA concentrations were raised in 78/100 (78%), 60/100 (60%) and 89/100 (89%) of the untreated CD patients respectively. The IgA EmA was positive in 86/100 (86%) (**Table 5.2**). IgA anti-tTG antibody concentrations were significantly higher in untreated CD patients (median: 9740 units/ml; range 300-89400 units/ml) than in all other control groups (Mann-Whitney test: $p < 0.0001$), except for the DH patients (Mann-Whitney test: $p = 0.59$) (**Figure 5.1**). Of the 22 untreated CD patients with normal serum IgA anti-tTG antibody and total IgA concentrations, 11 were IgA EmA positive and the IgA AGA and IgG AGA concentrations were raised in 6 and 15 patients respectively. Three untreated CD patients had borderline IgA anti-tTG concentrations of 2220 units/ml, 2340 units/ml and 2510 units/ml. The IgA EmA was negative in 13/100 (13%) and equivocal in 1/100 (1%). The IgA anti-tTG antibody concentrations were raised in 3 of these patients.

Table 5.2: Serological Results of Coeliac Disease & Dermatitis Herpetiformis Patients, Disease Controls & Volunteers

Disease Category	IgA AGA Positive	IgA EmA Positive	IgA tTG Positive	Median IgA tTG (units/ml)
Untreated CD n=100	60 (60%)	86/100 (86%)	78/100 (78%)	9740
DH n= 25	13 (52%)	17/25 (68%)	20/25 (80%)	6670
Treated CD n=82	14 (17%)	0/82	1/82 (1%)	635
Non-CD Controls n=65	9 (14%)	0/70	2/65 (3%)	300
UC n=82	13 (16%)	0/82	2/82 (2%)	730
Crohn's Disease n=80	13 (16%)	1/80 (1%)	2/80 (2%)	840
IBS n=36	2 (6%)	0/36	1/36 (3%)	560
Infective/Idiopathic Diarrhoea n=15	3 (20%)	0/15	2/15 ^a (13%)	1000
Other Diagnoses n=46	8 (17%)	0/46	3/46 ^b (7%)	760
Healthy Volunteers n=29	0	1/29 (3%)	2/29 (7%)	410

Two untreated CD patients with SIgAD have been excluded. Five patients indicated by the symbols, ^a and ^b had raised serum IgA anti-tTG antibody concentrations. The diagnoses of these patients were giardiasis, malabsorption following intestinal bypass surgery for obesity, rectal tubular adenoma, pneumatosis cystoides intestinalis and Cronkhite-Canada syndrome.

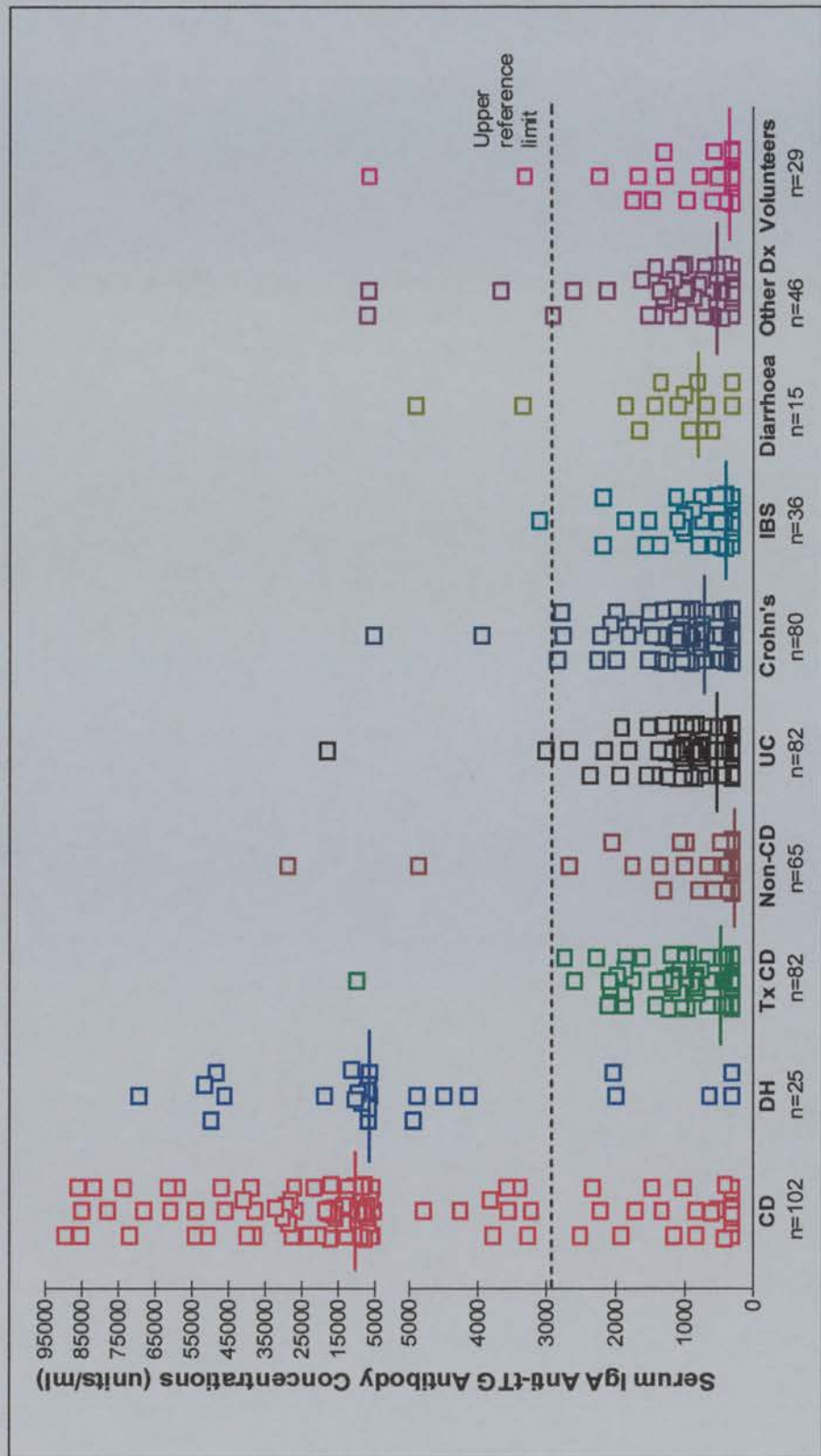


Figure 5.1: Serum IgA anti-tTG antibody concentrations in CD, DH, disease controls & healthy volunteers are shown. The horizontal lines indicate the median values. Serum IgA anti-tTG is significantly higher in untreated CD and DH patients than in all other groups (Mann Whitney test: $p < 0.0001$).

The sensitivity and specificity of the serological tests in these 100 CD patients are shown in **Table 5.3**. The sensitivity of the IgA anti-tTG antibody ELISA would increase from 78% (78/100) to 87% (75/86) if only the IgA EmA positive patients had been included. The IgA EmA assay had the highest specificity, but this finding may have been biased by the technique used in selecting the group of non-CD patients. As discussed in **section 5.2**, these patients were selected on the basis of having a negative IgA EmA and normal duodenal mucosal histology. The specificities of the IgA AGA and IgG AGA assays were reduced because of the high false positive rate of 9/65 (14%) and 22% (14/65) in the non-CD control patients. The combination of serological tests enhanced the detection of untreated CD (**Table 5.4**). The highest sensitivity in diagnosing CD of 100% resulted from the combinations of IgA anti-tTG with IgA and IgG AGA. The combination of IgA EmA with IgG AGA (\pm IgA AGA) produced a sensitivity of 96%. The IgA anti-tTG and IgA EmA assays had the highest and comparable PPVs of 98 and 100% respectively.

Table 5.3: Sensitivity & Specificity of Serological Tests using In-House Assays

Serological Tests	Sensitivity (n=100)	Specificity (n=65)	Positive Predictive Value	Negative Predictive Value
IgA Anti-tTG	78 (78%)	63 (97%)	78/80 (98%)	63/85 (74%)
IgA EmA	86 (86%)	65 (100%)	86/86 (100%)	65/79 (82%)
IgA AGA	60 (60%)	56 (86%)	60/69 (87%)	56/96 (58%)
IgG AGA	87 (87%)	51 (78%)	87/101 (86%)	51/64 (80%)

Table 5.4: Sensitivity of Serological Tests in Combination

Serological Tests	Sensitivity (n=100)	Positive Predictive Value
IgA tTG	78%	98%
IgA tTG + IgA AGA	84%	90%
IgA tTG + IgG AGA	93%	85%
IgA tTG + IgA AGA + IgG AGA	100%	83%
IgA EmA	86%	100%
IgA EmA + IgA AGA	88%	91%
IgA EmA + IgG AGA	96%	88%
IgA EmA + IgA AGA + IgG AGA	96%	84%

The 2 untreated CD patients with SIgAD have been excluded from the sensitivity calculations. Combining serological methods increases the sensitivity of serological methods, especially when both IgA and IgG-class antibodies are used.

5.4.3 Serological Results in Dermatitis Herpetiformis Patients

Of the 25 DH patients studied, 19 patients had evidence of villous atrophy and 2 patients had raised IELs only all consistent with a diagnosis of CD. The 4 remaining patients all had entirely normal small intestinal histology. Serum IgA anti-tTG antibody concentrations were raised in 20/25 (80%) of these patients and the levels were significantly higher than in the treated CD, non-CD, disease and healthy controls (Mann-Whitney test: $p < 0.0001$). Of the 21 DH patients with histological abnormalities, 17 (81%) patients had a raised serum IgA anti-tTG antibody concentration. The IgA EmA was positive in 17/25 (68%) patients and the IgA AGA and IgG AGA concentrations were raised in 13/25 (52%) and 22/25 (88%) of patients respectively. The serological results of the patients with normal histology or only mild abnormalities are given in **Table 5.5**.

Table 5.5: Serological Results of Dermatitis Herpetiformis Patients with Normal Small Intestinal Histology or Raised IELs Only

Patient	Age / Gender	Histology	IgA AGA (units/ml)	IgG AGA (units/ml)	IgA EmA	IgA Anti-tTG (units/ml)
1	32 M	Raised IELs	6	50	Negative	635
2	50 M	Raised IELs	23	55	Negative	2030
3	72 F	Normal	13	184	Positive	1995
4	59 M	Normal	30	16	Negative	6350
5	65 F	Normal	6	61	Positive	6450
6	53 M	Normal	48	120	Positive	4136

Serological and histological results of the 6 prospectively diagnosed DH patients are given in **Table 5.6**. The serum IgA anti-tTG antibody concentrations were raised in all 6 patients, and the IgA EmA was negative in 2 patients. Patient 1 was an asymptomatic 69 year old retired joiner, who presented to the dermatologists with a pruritic rash. His duodenal biopsies showed PVA and he was commenced on a GFD in addition to dapsone therapy. Patient 2 was a 72 year old man who presented with a 4 month history of profuse diarrhoea, and the pruritic skin rash appeared after the diagnosis of CD was made. Patient 3 was a 51 year old nursing auxiliary, who presented with a skin rash, but also had a history of recurrent iron deficiency anaemia, intermittent diarrhoea, abdominal discomfort, and weight loss of over 2 stones over a 3 year period. Duodenal biopsies showed PVA and her abdominal symptoms markedly improved following treatment with a GFD. Patient 4 was an asymptomatic 71 year old retired joiner whose duodenal biopsies showed PVA. His skin rash responded well to treatment with a GFD and dapsone. Repeat duodenal biopsies were performed in this patient following dietary treatment because his serum IgA EmA was negative. This showed complete normalisation of his villous architecture.

Patient 5 was a 53 year old building surveyor with essential thrombocythaemia and pulmonary sarcoidosis, who was referred by the haematologists. At a routine clinic review, he gave a short history of a blistering skin rash, recurrent mouth ulceration and abdominal bloating, and was found to have a positive IgA AGA and EmA. A diagnosis of DH was histologically confirmed and dapsone was commenced, but he refused to follow a GFD. Patient 6 was a 59 year old crane driver, who was asymptomatic except for a 2 year history of a pruritic skin, which was confirmed to be DH. His duodenal biopsies were normal, and his skin lesions responded well to treatment with dapsone and a GFD.

Table 5.6: Prospectively Diagnosed Dermatitis Herpetiformis Patients

Patient	Age/ Gender	Serum IgA AGA (units/ml)	Serum IgG AGA (units/ml)	Serum IgA EmA	Serum IgA Anti- tTG (units/ml)	Duodenal Histology
1	69 M	42	177	Positive	4480	PVA
2	72 M	49	1070	Positive	4930	TVA
3	51 F	31	117	Positive	6670	PVA
4	71 M	53	243	Negative	8440	PVA
5	53 M	48	120	Positive	4135	Normal
6	59 M	30	16	Negative	6350	Normal

5.4.4 Serological Results in Treated CD Patients & Disease Controls

Serum IgA anti-tTG antibody concentrations were raised in 1/82 (1%) of treated CD patients, in 2/65 (3%) non-CD controls and in 10/259 (4%) of GI disease controls (**Table 5.2**). Serum IgA and IgG AGA concentrations were raised in 9/65 (14%) and 14/65 (22%) of non-CD controls with normal duodenal biopsies respectively. Elevated IgA AGA concentrations were also seen in 14/82 (17%) of treated CD patients and 39/259 (15%) of disease controls. The serum concentrations of IgA and IgG AGA in the groups studied are shown in **Figures 5.2** and **5.3** respectively.

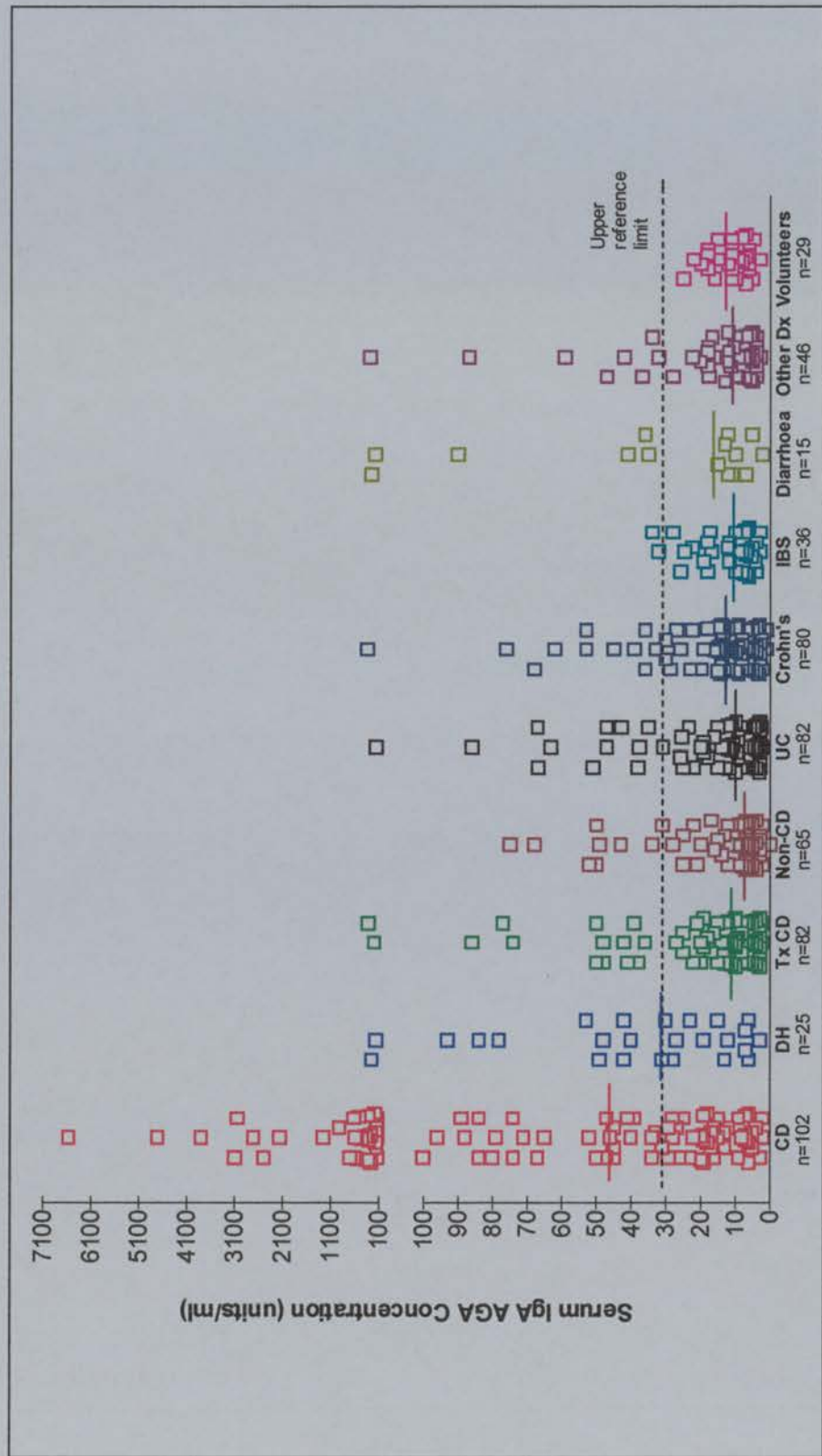


Figure 5.2: Serum IgA AGA concentrations in CD, DH, disease controls & healthy volunteers. The horizontal lines indicate the median values. Serum IgA AGA is significantly higher in untreated CD and DH patients than in all other groups (Mann Whitney test: $p < 0.0001$).

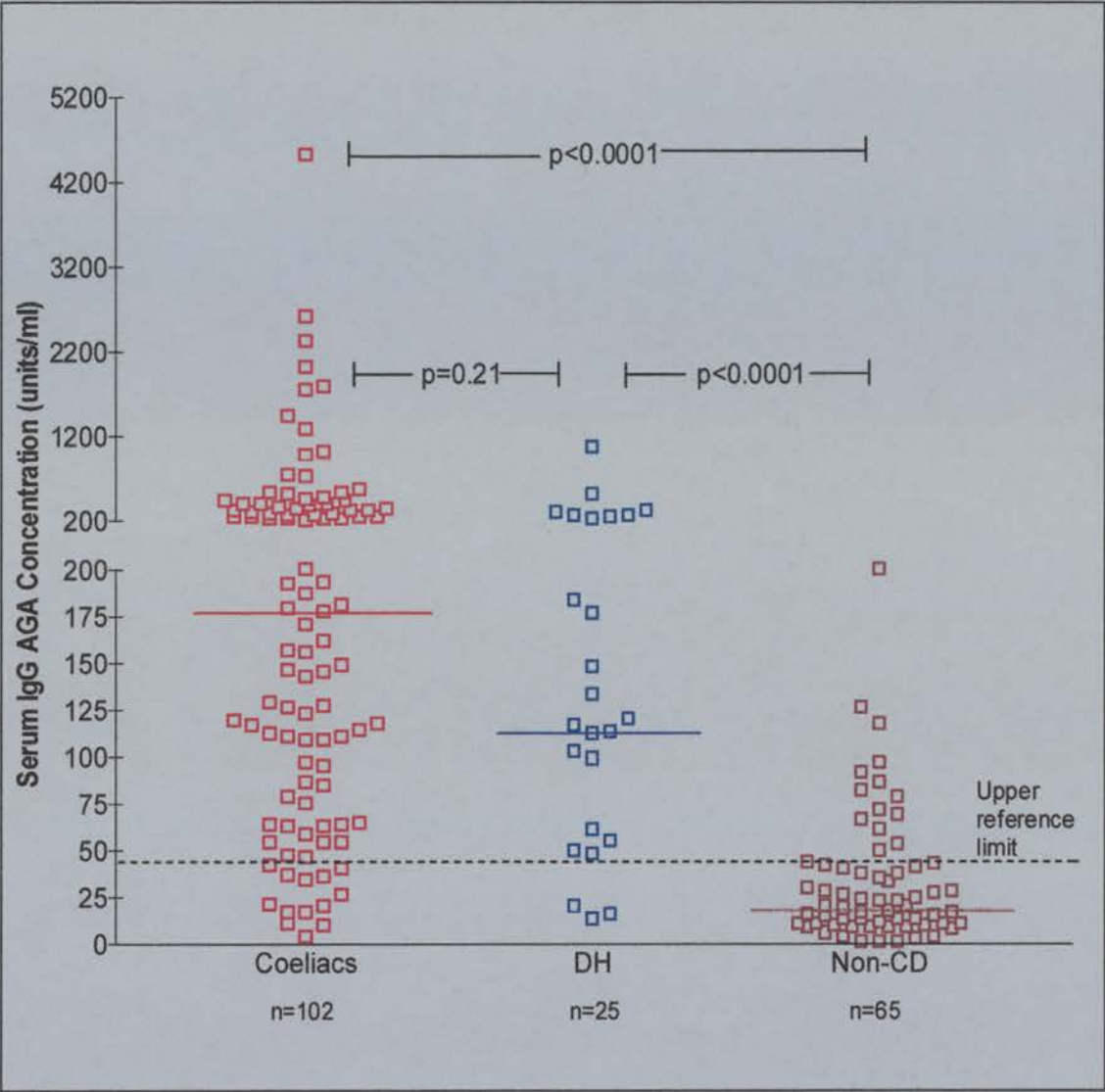


Figure 5.3: Serum IgG AGA concentrations in CD, DH & non-coeliac controls. The horizontal lines indicate the median values. The serum IgG AGA concentrations are significantly higher in untreated CD and DH patients than non-coeliac disease subjects (Mann-Whitney test: $p<0.0001$).

One of the 2 non-CD controls had a very high IgA anti-tTG antibody concentration of 29000 units/ml, but a negative IgA EmA. This 48 year old lady was being investigated for symptoms of diarrhoea and abdominal cramps, but was not thought to have CD as her duodenal biopsy was reported as normal. In retrospect, this lady had inadvertently been taking a low-gluten containing diet at the time of her intestinal biopsy and serum sampling. This patient initially agreed to undergo a supervised gluten challenge and repeat small intestinal biopsies, but failed to attend. The other non-CD control had an IgA anti-tTG antibody concentration of 4860 units/ml. This 40 year old lady had presented with symptoms of abdominal discomfort, loose stools alternating with constipation and dyspepsia. She had a normal upper endoscopy and barium enema examination. Her symptoms were thought to be due to functional bowel disease.

One 60 year old male patient with UC had a raised IgA anti-tTG concentration of 18100 units/ml, but the IgA EmA was negative and the AGA concentration was within reference limits. This individual has not undergone small intestinal biopsy. One 58 year old female Crohn's disease patient, with active ileal disease, had a raised IgA anti-tTG antibody concentration of 5260 units/ml and a positive IgA EmA, but a normal duodenal biopsy including IEL count. Interestingly, subsequent repeat serological tests have all been negative.

5.4.5 Serological Results in Healthy Volunteers

Serum IgA anti-tTG antibody concentrations were raised in 2/29 (7%) of healthy volunteers (**Table 5.2**). Two healthy male volunteers had raised IgA anti-tTG antibody concentrations of 6495 units/ml and 3315 units/ml. Only the former was IgA EmA positive, and subsequent duodenal biopsies showed PVA consistent with untreated CD. In retrospect, this "asymptomatic" member of the medical staff reported intermittent diarrhoea and dyspepsia. There was no family history of CD, but his sister is currently being investigated for anaemia. Subsequent testing 3 months after therapy with a GFD

showed that the IgA EmA had become negative. The IgA anti-tTG antibody concentration was not performed on the second sample as it was obtained outwith the study period. The EmA was negative and the IgA AGA concentration was within normal limits in the other volunteer who has not undergone duodenal biopsy. None of the healthy volunteers had a raised serum IgA AGA concentration.

5.4.6 Serological Results Following Treatment with a GFD

Of the 53 prospectively diagnosed untreated CD and DH patients, serum samples were obtained from 32 patients after a median duration of 5 months (range 2-12 months) treatment with a GFD. However, the pre-treatment IgA anti-tTG antibody concentrations were raised in only 23 (72%) of these patients (median 15700 units/ml; range 4480-81600 units/ml). All of these patients had a symptomatic improvement following gluten withdrawal. IgA anti-tTG antibody concentrations remained negative following gluten withdrawal in the other 9 (28%) patients and therefore only the data for the 23 patients (17 F: 6 M; age range 27-71 years - median 53 years) are shown. Pre-treatment IgA EmA antibodies were positive in 20/23 (87%) and the IgA AGA concentration was raised in 21/23 (91%) patients.

There was a significant reduction in the IgA anti-tTG antibody concentrations before (median 15700 units/ml; range 4480-81600 units/ml) and after (median 1620 units/ml; range 615-7305 units/ml) therapy with a GFD (Mann-Whitney test, $p < 0.0001$) (**Figure 5.4**). The antibody concentration had returned to normal in 18/23 (78%) patients within 12 months. Two patients had consented to attend regularly for close monitoring of the IgA anti-tTG antibody concentrations after starting their GFD. The rates of decline in their antibody concentrations are shown in **Figure 5.5a & 5.5b**. The serological results and initial duodenal biopsy histology of the 5 patients with a persistently raised IgA anti-tTG antibody concentration after dietary therapy are shown in **Table 5.7**. The IgA EmA and IgA AGA had both returned to within reference limits in the two patients

(patients 1 & 2) with a borderline raised IgA anti-tTG antibody concentration, but remained abnormal in the other 3 patients in whom the IgA anti-tTG antibody concentrations were markedly raised.

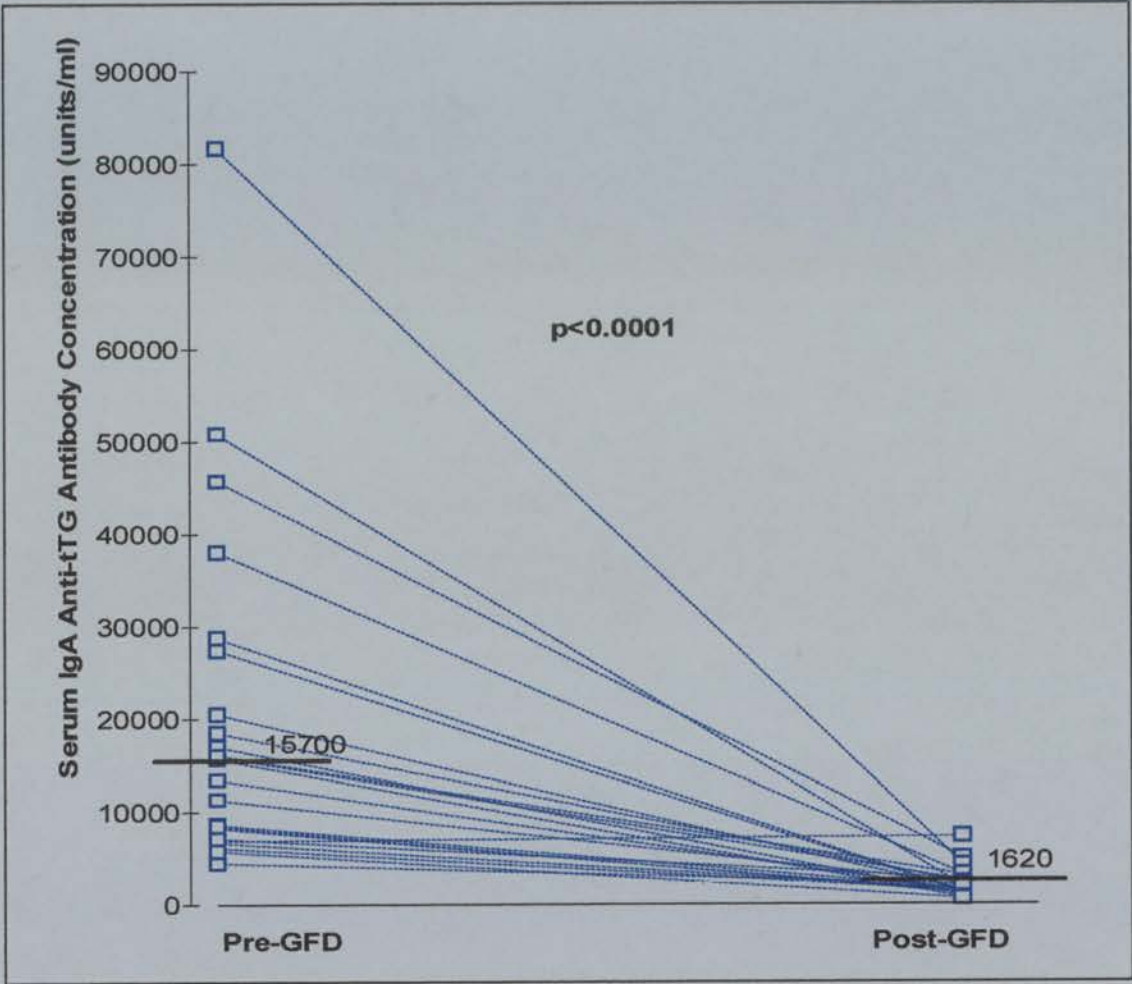


Figure 5.4: IgA anti-tTG antibody concentrations in 23 CD patients before & after therapy with a GFD. The horizontal lines indicate the median values. There was a significant reduction in the concentration of serum IgA anti-tTG antibodies following gluten withdrawal (Mann-Whitney test: $p < 0.0001$).

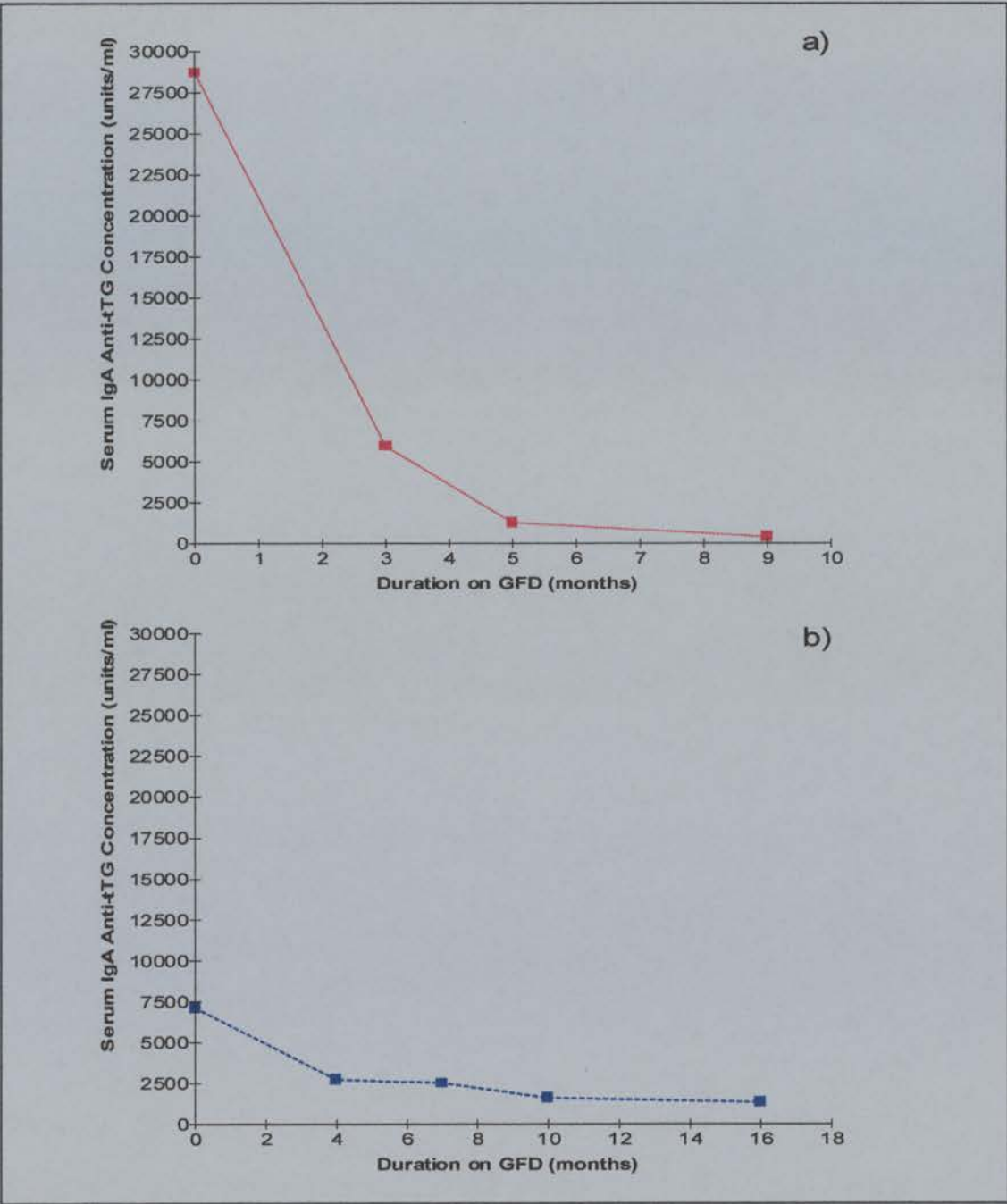


Figure 5.5a & b: Decline in IgA anti-tTG antibody concentrations in two CD patients

Table 5.7: Serological Results of the Coeliac Disease Patients with Raised IgA Anti-tTG Antibody Concentrations after treatment with a Gluten Free Diet

	Duration of GFD (months)	Pre-GFD IgA Anti-tTG (units/ml)	Post-GFD IgA Anti-tTG (units/ml)	Pre-GFD IgA EmA	Post-GFD IgA EmA	Pre-GFD IgA AGA (units/ml)	Post-GFD IgA AGA (units/ml)
1	6	16100	3145	Pos.	Neg.	84	24
2	2	38000	3215	Pos.	Neg.	178	28
3	8	81600	4235	Pos.	Equiv.	4694	40
4	11	45700	5040	Pos.	Pos.	2695	>100
5	3	6880	7305	Equiv.	Equiv.	583	>100

Following gluten withdrawal, the IgA EmA had become negative in 17 (74%) of the 23 patients. Four of the 6 patients with a continuing positive IgA EmA, had been on a GFD for less than 5 months **Table 5.8**. In addition, the initial antibody titre was very high ($\geq 1/50$) in 4 of these patients. The IgA anti-tTG antibody and IgA AGA concentrations were raised in 1 and 5 of these subjects respectively. Although similar numbers of the post-treatment CD patients had normalised their IgA anti-tTG antibody concentrations and IgA EmA, there were 9 discrepancies between these antibody results as shown in **Table 5.9**.

Table 5.8: Serological Results of the Coeliac Disease Patients with a Positive IgA EmA after treatment with a Gluten Free Diet

Patient	Duration of GFD (months)	Pre-GFD IgA Anti-tTG (units/ml)	Post-GFD IgA Anti-tTG (units/ml)	Initial IgA EmA titre	Pre-GFD IgA AGA (units/ml)	Post-GFD IgA AGA (units/ml)
1	2	8250	1295	1/200	545	61
2	3	6130	1405	1/10	3025	>100
3	5	18500	2340	1/10	22	12
4	2	16000	2550	1/50	310	38
5	4	7120	2720	1/100	39	42
6	11	45700	5040	1/100	2695	>100

Table 5.9: Discrepancies between IgA Anti-tTG Antibody Concentrations & IgA EmA in Coeliac Disease Patients Following Treatment With A Gluten Free Diet

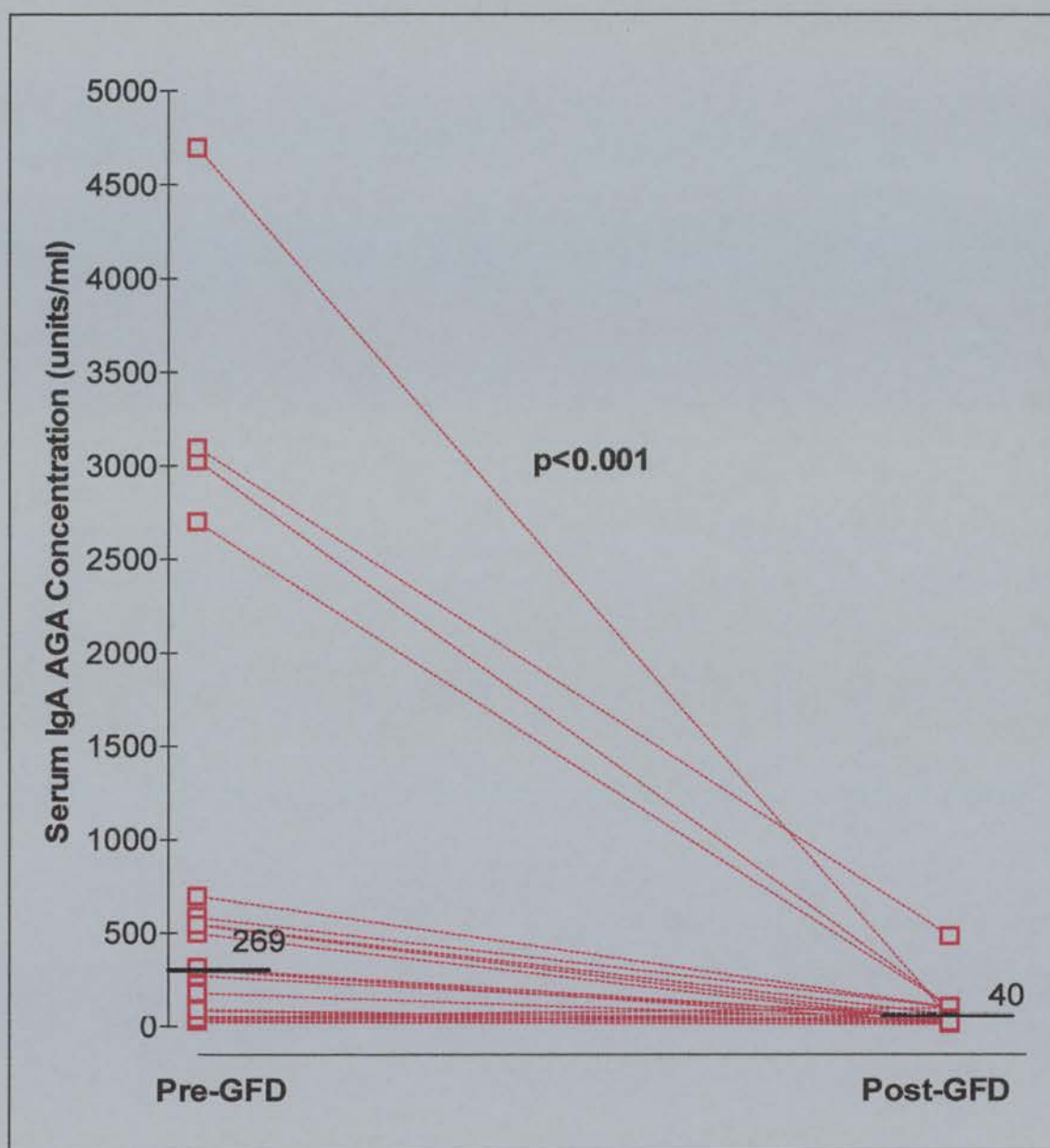
Patient	Initial Histology	Duration of GFD	IgA EmA	IgA Anti-tTG (units/ml)	IgA AGA (units/ml)
1	PVA	5	Positive	1295	61
2	PVA	4	Positive	1405	>100
3	Severe PVA	5	Positive	2340	12
4	STVA	7	Positive	2550	38
5	STVA	3	Positive	2715	42
6	PVA	6	Negative	3145	24
7	Severe PVA	3	Negative	3215	28
8	PVA	3	Equivocal	4235	40
9	STVA	11	Equivocal	7305	>100

During the treatment period, there was a significant decline in the IgA AGA concentrations (pre-treatment median 269 units/ml; range 22-4694 units/ml, and post-treatment median 40 units/ml; range 10-480 units/ml) (Mann-Whitney test, $p<0.001$) **Figure 5.6.** IgA AGA concentrations had declined in all but 4/23 (17%) patients, and returned to within reference limits in 9/23 (39%) patients. Post-treatment IgG AGA concentrations were not measured. Repeat duodenal biopsies were not routinely

performed following an interval on a GFD, unless the initial IgA EmA was negative (see section 6.3).

5.4.7 Comparison between Serum IgA Anti-tTG Antibodies & IgA EmA

The IgA EmA was positive in 105 samples: untreated CD (n=86), DH (n=17), Crohn's disease (n=1) and healthy volunteer (n=1). The IgA EmA was negative in a total of 457 samples. Serum IgA anti-tTG antibody concentrations were significantly higher in the IgA EmA positive (median 11900 units/ml; range <300 - 89400 U/ml) than the IgA EmA negative (median 620 units/ml; range 300 - 18100 units/ml) serum samples (Mann Whitney test: $p < 0.00001$) (**Figure 5.7**). The IgA anti-tTG antibody concentrations were compared with the corresponding IgA EmA titres in 37 of the IgA EmA positive untreated CD and DH patients in whom EmA titres were determined. This showed that IgA anti-tTG antibody concentrations correlated well with EmA titres ($r = 0.54$; $p < 0.0001$) (**Figure 5.8**). The concordance rate between IgA anti-tTG and IgA EmA was 85%.



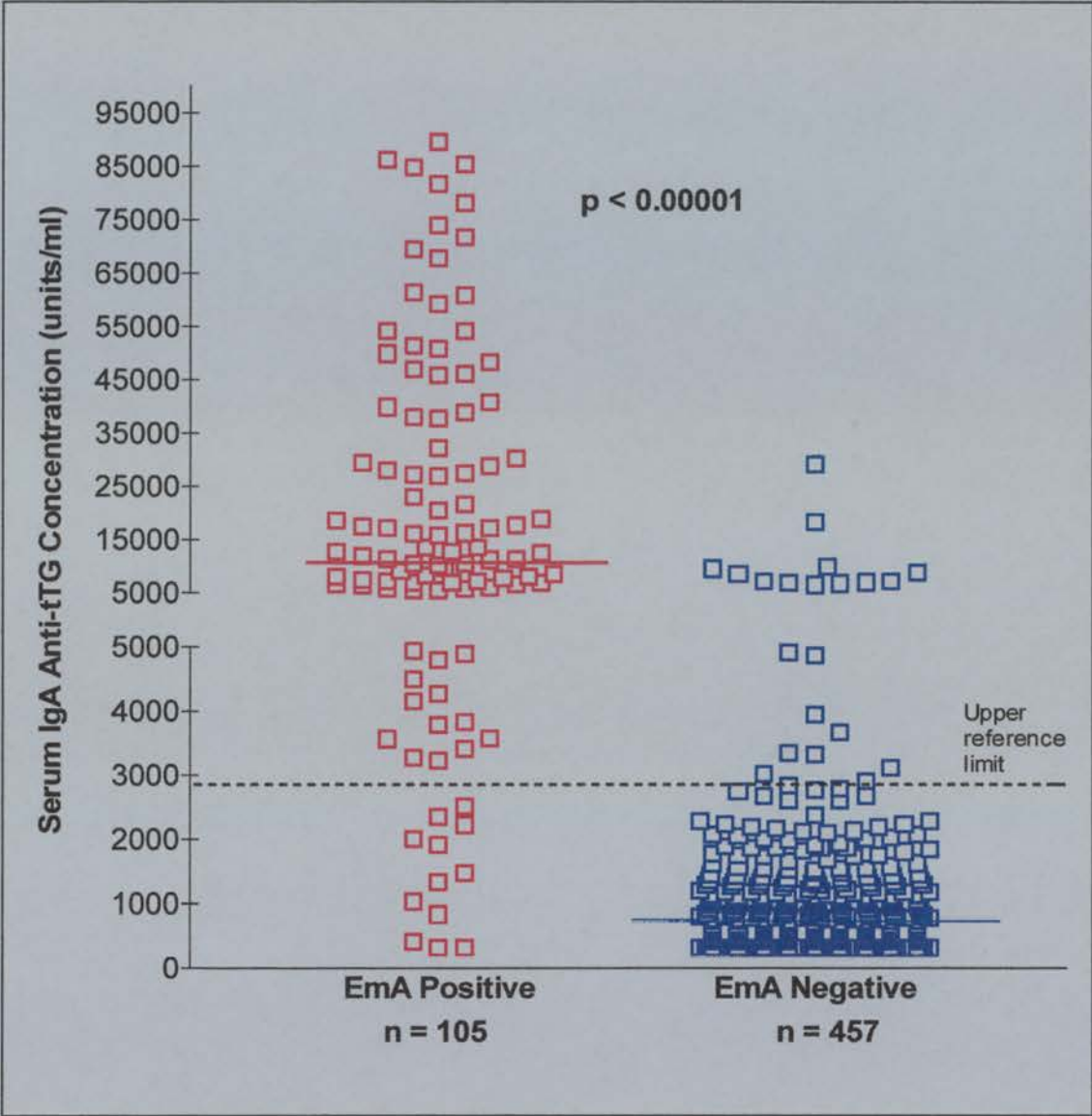


Figure 5.7: Serum IgA AGA concentrations in IgA EmA positive & negative CD patients. The horizontal lines indicate the median values. The concentration of serum IgA anti-tTG antibodies was significantly higher in EmA positive than negative subjects (Mann-Whitney test: $p < 0.00001$).

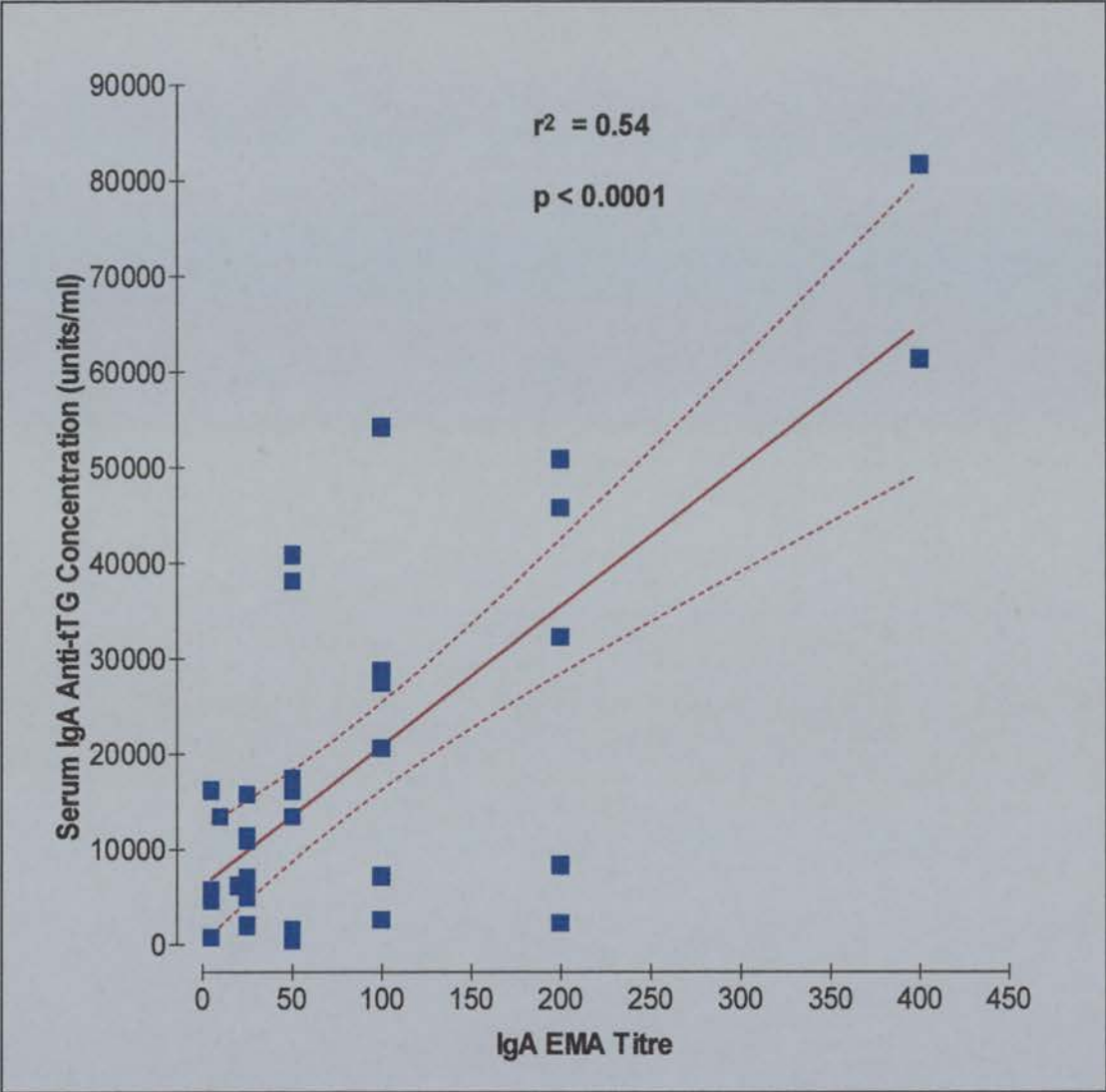


Figure 5.8: Correlation between serum IgA anti-tTG antibody concentrations & IgA Ema antibody titre

5.5 Discussion

Serological testing for the IgA anti-tTG antibodies is useful, but is not superior to, IgA EmA in screening for CD and DH. IgA anti-tTG antibody concentrations were significantly higher in untreated CD and DH than in treated CD, non-CD and GI disease, or healthy controls. IgG anti-tTG antibodies were not measured in the 2 patients with SIgAD as the assay performance was very disappointing for the reasons described in **section 3.4.7**. Others however, have shown that IgA anti-tTG antibody concentrations were normal, but IgG anti-tTG antibody levels raised in of the untreated CD patients with SIgAD tested (n=14) (Sulkanen et al. 1998b). The finding of a positive IgA anti-tTG antibody and IgA EmA result in a healthy “asymptomatic” volunteer with PVA supports the observation subtle presentations of CD may be easily missed (Swinson and Levi, 1980; Catassi et al. 1994). The high IgA anti-tTG antibody concentrations found in untreated CD patients is probably because dietary gliadin is known to be an excellent substrate for tTG (Bruce et al. 1985), and because the concentration of gliadin is highest in the proximal intestine. The likely mechanism for the production of anti-tTG antibodies was discussed in **section 2.4.3**.

In the 100 CD patients with normal serum total IgA, the sensitivity and specificity of the IgA anti-tTG antibodies and IgA EmA were 78% and 97%, and 86% and 100% respectively. The PPVs were comparable at 98% and 100% respectively, but the NPV of IgA EmA (82%) was better than that of the IgA anti-tTG antibodies (74%). The IgA anti-tTG antibodies were raised in 3 IgA EmA negative untreated CD patients, but failed to detect 11 IgA EmA positive patients. Others have also found discrepancies between IgA anti-tTG antibody and IgA EmA results (Dieterich et al. 1998; Troncone et al. 1999). Dieterich *et al.* reported that the IgA anti-tTG antibody concentration was normal in 4 EmA positive CD patients, and was raised in 10 EmA negative samples (Dieterich et al. 1998). This may in part be due to the difference sources of the tissue

substrates used in the two assays. The slightly lower specificity of the IgA anti-tTG antibody assay than the IgA EmA test, may in part be due to the use of the non-human source of tTG (from guinea pig liver) in comparison to the use of HUC substrate in the EmA assay. Despite this, like others (Dieterich et al. 1997; Sulkanen et al. 1998b; Dieterich et al. 1998), we found a good correlation between IgA anti-tTG antibody concentrations and IgA EmA titres. The sensitivity and specificity of the IgA AGA and IgG AGA were 60% and 86%, and 87% and 78% respectively. A raised IgA AGA or IgG AGA had comparable PPVs of 87% and 86% respectively, which was much lower than that of the IgA anti-tTG antibodies or IgA EmA.

Positive IgA anti-tTG, IgA EmA and IgA AGA antibodies were found in 4% (10/259), 0.4% (1/259) and 15% (39/259) of the GI disease controls respectively. All 3 serological markers were positive in one female Crohn's disease patient with active ileal disease, but with normal duodenal histology. Unfortunately, fresh biopsies were not available from this patient to determine the CD3 and γ/δ IEL counts. Interestingly, all of these markers had normalised 2 years later when another serum sample was obtained. It is possible that this individual has latent CD. Others have not found raised IgA anti-tTG antibody concentrations in any IBD patients (Sulkanen et al. 1998b; Dieterich et al. 1997; Dieterich et al. 1998) but did find high levels in other GI controls (Sulkanen et al. 1998b). The finding of elevated IgA anti-tTG antibody concentrations in other GI disorders may be explained by the wide distribution of the enzyme throughout the gut (D'Argenio et al. 1988; Patel et al. 1985).

Recent reports on the sensitivity and specificity of the IgA anti-tTG assay (using the same guinea pig liver enzyme) have ranged from 85-98% and 90-98% (Sulkanen et al. 1998b; Dieterich et al. 1998; Lock et al. 1999b; Troncone et al. 1999; Biagi et al. 1999a). Unlike these studies however, the CD patients in this study were biopsied on the basis of clinical suspicion and were not selected exclusively by positive serological

tests. This approach produced a high prevalence of IgA EmA negative untreated CD patients of 16% (16/102), of whom only 2 had SIgAD. The high proportion of IgA EmA negative CD patients reduced the IgA anti-tTG antibody ELISA sensitivity as it improved from 78% (78/100) to 87% (75/86) when the IgA EmA negative CD patients were excluded. The sensitivities of IgA anti-tTG antibodies and IgA EmA improved when used in combination with IgG AGA test giving sensitivities of 93% and 96% respectively. The addition of IgG AGA however resulted in a reduction in specificity and in the PPV.

The Maki method for measuring IgA anti-tTG antibodies was described in **section 3.1.13**. The main difference from our method was that they used the calcium-activated enzyme. The sensitivity of the IgA anti-tTG antibody ELISA described by this group was 95% (129/136), which was markedly better than in our assay, but only 10 (7.4%) of their untreated CD patients had a negative IgA EmA (Sulkanen et al. 1998b). The number of EmA negative CD patients has undoubtedly influenced our assay sensitivity, even allowing for the differences between assay methodologies. Reassuringly, the specificities using our assay and in the Maki study were comparable at 97% and 94% respectively, and like us, they too found raised IgA anti-tTG antibody concentrations in their disease controls (Sulkanen et al. 1998b). Other groups whose ELISA method was very similar to our method (with respect to the exclusion of calcium from the coating stage and the use of a 1% BSA block), have found an assay sensitivity and specificity of 85% and 90% (Biagi et al. 1999a), and 84.8% and 91.1% (Bazzigaluppi et al. 1999) respectively. In these two studies however, the IgA EmA was positive in all (Biagi et al. 1999a) and in 97% (Bazzigaluppi et al. 1999) of the untreated CD patients. Therefore, the assay performance is very comparable to the findings in this study if only the EmA positive CD patients are included.

Recently, a radio-binding assay using human recombinant tTG has been shown to compare well with EmA findings with a sensitivity of 99.1% (111/112) and specificity of 95.7% (Bazzigaluppi et al. 1999). This technique was shown to be superior to the IgA anti-tTG ELISA using guinea pig liver tTG dissolved in PBS at pH 7.3 (also without calcium and blocked with BSA) which had a sensitivity of 85% and specificity of 91% in the same group of patients (Bazzigaluppi et al. 1999). These latter findings compare well with our results in IgA EmA positive patients using a similar method except for the coating pH. The use of human recombinant tTG appears to be superior to the guinea pig liver tTG, with reported sensitivities and specificities of 97% and 100%, and 90% and 99% respectively (Sblattero et al. 1999). Similar findings have been observed with the use of HUC instead of MO as the tissue substrate in the EmA assay (Bottaro et al. 1997; Sategna-Guidetti et al. 1997). Although our current IgA anti-tTG antibody assay is not superior to the IgA EmA test, its performance is likely to improve in the future when a human recombinant tTG becomes commercially available.

In accordance with the revised ESPGAN guidelines on the diagnosis of CD (Walker-Smith et al. 1990), repeat biopsies were only performed in patients with continuing symptoms despite adequate dietary compliance, or those who had a negative IgA EmA at presentation. Without repeat biopsies to allow comparison with the serology results, this study was unable to determine if IgA anti-tTG antibodies were better than the IgA EmA or IgA AGA in monitoring gluten withdrawal. Of note however, all of these patients improved after a GFD was started. During the study period however, the number of patients in whom the IgA anti-tTG antibody concentrations and IgA EmA had normalised were very similar (78% and 74% respectively). In contrast, the IgA AGA concentrations had returned to within reference limits in only 9 of the 23 CD patients studied.

The changes in serology following dietary therapy and the relative merits of the different markers used in monitoring gluten withdrawal have been discussed previously in **section 2.8**. It is well established that the concentrations of IgA AGA decline (de Lecea et al. 1996; Scott et al. 1990) and the IgA EmA returns to normal (Hallstrom, 1989) after dietary therapy. IgG AGA concentrations remain raised for many months (Savilahti et al. 1983). The seroconversion to a negative EmA has been found to correlate well with a histological remission (Ladinser et al. 1994). Others however, have found that the IgA EmA may not correspond to histological findings, as in some patients the EmA may remain positive despite histological remission (Usselman and Loft, 1996; Volta et al. 1991), or is negative in the presence of persistent jejunal abnormalities (Valentini et al. 1994). The presence of a negative IgA EmA appears to correspond more closely with histology than does the AGA (Lerner et al. 1994). In this study we showed that the serum IgA anti-tTG antibody concentrations progressively decline after introduction of a GFD. This is in agreement with another report (Dieterich et al. 1997).

There is still limited work however, on the changes in IgA anti-tTG antibody concentrations after treatment or following gluten challenge. It has previously been demonstrated that IgA anti-tTG antibody levels decline after gluten restriction (Dieterich et al. 1997; Sulkanen et al. 1998b), and that there is a good correlation with decreasing titres of IgA EmA (Dieterich et al. 1997). It has also been shown that IgA anti-tTG antibody levels are significantly lower in treated than in untreated CD patients (Dieterich et al. 1998; Troncone et al. 1999), but few groups have prospectively monitored the change in antibody levels after the start of a GFD. Sulkanen *et al.* obtained follow-up serology and biopsies from 38 biopsy-proven CD patients after a median duration of 48 months (range 14-186 months), and from 18 patients following a gluten challenge (Sulkanen et al. 1998b). They found that 61% (23/38) of CD patients showed a negative anti-tTG antibody seroconversion following gluten withdrawal, and

that all 18 patients undergoing gluten challenge had raised anti-tTG antibody concentrations at the time of mucosal relapse (Sulkanen et al. 1998b).

There is a need for reliable, less invasive and less expensive means of diagnosing CD, but there is a risk that serology may not identify a proportion of these patient. Objective serological methods would be advantageous over the observer-dependent EmA assay, but our current IgA anti-tTG antibody ELISA is less sensitive and specific. There is scope for improving the assay and this will be discussed in the final chapter. The performance of the IgA anti-tTG antibody assay in the literature is better because they have included IgA EmA positive patients predominantly. As it is very important to recognise EmA negative CD patients, the next chapter describes the clinical and laboratory characteristics of the prospectively diagnosed IgA EmA negative CD patients recruited in this study.

CHAPTER 6: CHARACTERISTICS OF IgA ENDOMYSIUM NEGATIVE COELIAC DISEASE PATIENTS

6.1 Introduction

In the last chapter, the IgA EmA test was shown to have the best overall assay performance, yet it still failed to detect all of the gluten-sensitive individuals. The sensitivity and specificity of IgA EmA in the 100 untreated CD patients studied (with normal serum total IgA) were 86% and 100% respectively. This compares favourably with the corresponding values in the literature of 74-100% and 96-100% (Hallstrom, 1989; Volta et al. 1991; Kolho and Savilahti, 1997; Valdimarsson et al. 1996; Cataldo et al. 1995), respectively (see **Table 2.2**). It is known that the sensitivity of the IgA EmA test depends upon whether only sero-positive patients are biopsied for confirmation, or whether patients are biopsied on the basis of clinical suspicion. A positive IgA EmA may also depend on age, the severity of the mucosal lesion (Rostami et al. 1999a; Rostami et al. 1998), the length of intestine involved (Mulder et al. 1998), as well as on genetic factors (Cataldo et al. 1995). Selective IgA deficiency is known to be associated with a 10-fold increased risk of CD compared to the general population (Collin et al. 1992; Cataldo et al. 1999). Relying on IgA-based serological testing to diagnose CD may produce misleading results in this group of patients. Even after excluding SIgAD, there is still a definite false-negative rate for the IgA EmA test (Rostami et al. 1999a; Rostami et al. 1998; Dick et al. 1969).

The 1969 protocol for the diagnosis of CD proposed by the ESPGAN, required an initial characteristic biopsy while on a normal diet, histological improvement on gluten withdrawal and deterioration following gluten challenge (Meeuwisse, 1970). These

guidelines were later simplified (Walker-Smith et al. 1990), such that the diagnosis of CD was supported by a characteristic initial biopsy followed by a definite, reasonably rapid clinical remission on a strict GFD with relief of all symptoms (Walker-Smith et al. 1990). Positive serological tests, together with their disappearance in line with a clinical response, support the diagnosis. The finding of occasional false positive and negative results meant that the diagnosis could still not be established exclusively on the basis of positive serology (Walker-Smith et al. 1990). Repeat small intestinal biopsies following dietary treatment or gluten challenge were no longer routinely required.

In this chapter, the clinical and serological characteristics of IgA EmA negative and positive CD are compared, and the role of additional serological and non-invasive tests in identifying these patients is discussed.

6.2 Patients

Prospectively Diagnosed Untreated CD & DH Patients:

Only the 53 prospectively diagnosed CD and DH patients (39 F: 14 M; age range 22-77 years - median 51 years) between October 1997 and April 1999 were investigated in this arm of the study. These 53 patients included the 49 CD patients and 4 DH patients with histologically confirmed CD discussed in **section 5.2**. All of these patients were consuming a normal gluten-containing diet at the time of serum sampling and endoscopic duodenal biopsy, and none of them were receiving immunosuppressants. Duodenal biopsies were performed on the basis of high clinical suspicion of CD and not exclusively after the finding of positive serological results. Clinical indications for investigation included diarrhoea, weight loss, abdominal pain, anaemia and/or macrocytosis. The small intestinal histology showed PVA in 27 patients, STVA in 25 patients and TVA in 1 patient. All patients had responded clinically to gluten withdrawal.

Non-CD Controls:

Control serum samples were obtained from the same 65 patients (46 F: 19 M; age range 17-90 years, median 45 years) investigated for suspected CD, but who subsequently were found to have normal duodenal mucosal histology described in **section 5.2**.

6.3 METHODS & STATISTICAL ANALYSIS

The clinical presentations, duration of symptoms and frequency of autoimmune diseases were recorded in these patients. The HLA-DQ2 typing was performed on 43 of the 53 CD patients studied using the technique described in **section 4.7**. For this analysis, all blood samples were obtained from patients during routine clinic visits and stored at 4°C until the test could be performed in a single batch. The dot blot results were interpreted blinded to the patient details.

All serum samples were analysed for IgA anti-tTG antibodies, IgA AGA, IgG AGA and IgA EmA using methods described in **sections 3.3, 4.2.3, 4.2.4 and 4.4.3** respectively. The upper reference limits of serum IgA anti-tTG, IgA AGA and IgG AGA were ≥ 2950 units/ml, ≥ 30 units/ml and ≥ 45 units/ml respectively. Serum total IgA was measured in all untreated CD patients to screen for SIgAD as part of the routine clinical chemistry laboratory service (reference range 1.01-4.23 g/l). Serum albumin and calcium concentrations (reference ranges 35-50 g/l and 2.1-2.6 mmol/l respectively) were also measured by the clinical chemistry laboratory. IgA/G/M-class ARA antibodies were determined in the IgA EmA negative CD patients only using the method outline described in **section 4.4.4**. An intestinal permeability test was performed in all patients, except the IDDM patients, using the method described in **section 4.8**. The normal L/R ratio was < 0.04 . All of these patients had endoscopic duodenal biopsies obtained and subsequently reported by members of the pathology department as part of the routine

National Health Service remit. Repeat duodenal biopsies were performed following treatment with a GFD in IgA EmA negative patients only.

The statistical methods applied were described in **section 4.9**. The χ^2 test was used to compare the frequency of symptoms and autoimmune diseases in EmA negative and positive CD patients. The Mann-Whitney test was used to compare the duration of symptoms, delay in diagnosis, as well as calcium and albumin concentrations of EmA negative versus positive CD patients. The sensitivities of the serological methods were calculated as the frequency of positive results in biopsy-proven CD patients while on a normal diet. Assay specificities were calculated as the frequency of negative results in the 65 non-CD control patients. The PPV and NPV of each serological method (except for ARA antibodies) were calculated.

6.4 Results

6.4.1 Clinical Characteristics of IgA EmA Negative Coeliac Disease Patients

Of the 53 untreated CD patients studied, the IgA EmA was negative in 12 patients and equivocal in 1 patient. This latter individual had a raised IgA anti-tTG antibody concentration, but was grouped with the EmA negative patients to permit comparison of results.

All of the IgA EmA negative CD patients presented with GI symptoms that prompted investigation, except for 1 DH patient. There was no significant difference observed in the presenting features of IgA EmA negative and positive CD patients (**Table 6.1**). The serum albumin concentrations were significantly lower however in the IgA EmA negative (median 38 g/l; range 26-43 g/l), than positive (median 40 g/l; range 28-47 g/l) CD patients (Mann-Whitney test, $p=0.03$). Autoimmune diseases were not seen more frequently in the EmA negative patients ($n=2$: 1 hypothyroidism, 1 collagenous colitis),

than in the EmA positive patients (n=4: 2 IDDM, 1 hyperparathyroidism, 1 Sicca syndrome) ($\chi^2 = 0.25$, p=0.616).

Table 6.1: Clinical Features of IgA EmA Negative Coeliac Disease Patients

Presenting Problems	IgA EmA Negative Patients n=13	IgA EmA Positive Patients n=40
Diarrhoea	10 (77%)	28 (72%)
Weight Loss	8 (62%)	23 (59%)
Abdominal Pain	7 (54%)	18 (46%)
Bloating	5 (38%)	18 (46%)
Fatigue	9 (69%)	30 (77%)
Anaemia	6 (46%)	22 (56%)
Hypoalbuminaemia	2 (15%)	2 (5.1%)
Hypocalcaemia	1 (7.6%)	2 (5.1%)

The clinical features of IgA EmA negative CD patients are not significantly different from EmA positive patients with the exception of a low serum albumin, which was observed more frequently in the EmA negative patients.

The duration of symptoms in the IgA EmA negative patients (median 24 months; range 1-300 months) was not significantly longer than for EmA positive patients (median 12 months; range 1-216 months) (Mann Whitney test, $p=0.26$). The interval between GI assessment and diagnosis of CD in IgA EmA negative patients (median 2 months; range 1- 72 months) was not significantly longer than for IgA EmA positive patients (median 1 month; range 1-48 months) (Mann Whitney, $p=0.08$).

Of note, the longest delays in diagnosis of CD were seen in 3 IgA EmA negative female patients, none of whom were IgA deficient. One 40 year old patient (patient 5 in **Table 6.4**) had first presented in 1984 with diarrhoea when investigations showed a normal jejunal biopsy and small bowel contrast study, but raised markers of bile acid malabsorption and vitamin B₁₂ deficiency. Worsening diarrhoea with weight loss were later investigated in 1998 by colonoscopy in an attempt to obtain ileal tissue to identify the cause of her ileal mucosal dysfunction. Histological examination showed ileal atrophy (**Figure 6.1a**). This prompted repeat investigation of her proximal small bowel by performing endoscopic duodenal biopsies. The biopsies showed patchy STVA (**Figure 6.1b**). All of the IgA and IgG-class serological markers were negative in this patient, but the L/R ratio was raised at 0.077. The serum albumin and calcium concentrations were within normal limits. Unfortunately, this patient failed to attend for numerous appointments for a repeat duodenal biopsy to assess her histological response, and was poorly compliant with her GFD. When following the diet, she did report a symptomatic improvement in her bowel habit. She has continued to receive therapy with cholestyramine and supplementation with vitamin B₁₂.

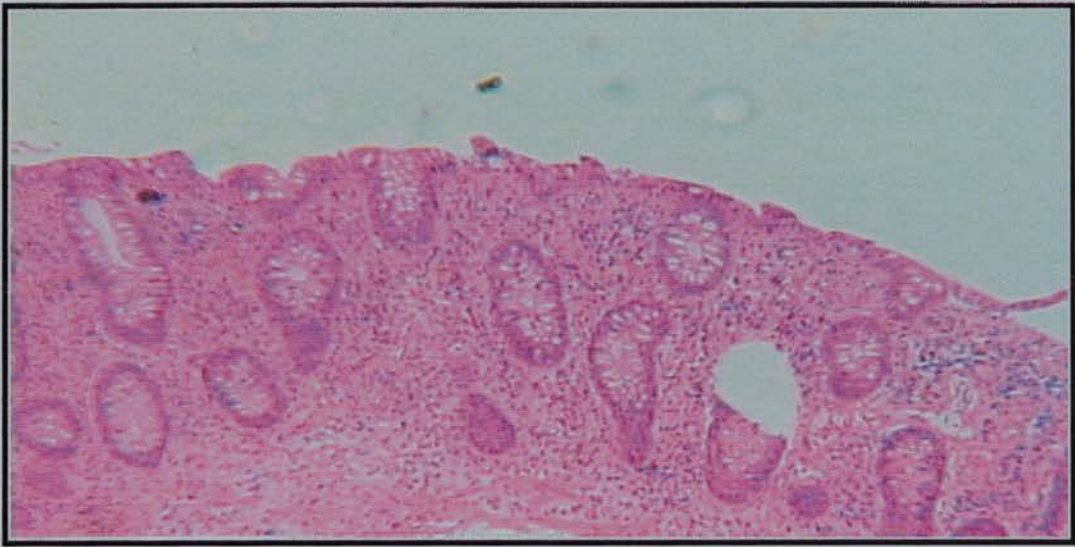


Figure 6.1a Ileal biopsy of an IgA EmA negative coeliac disease patient

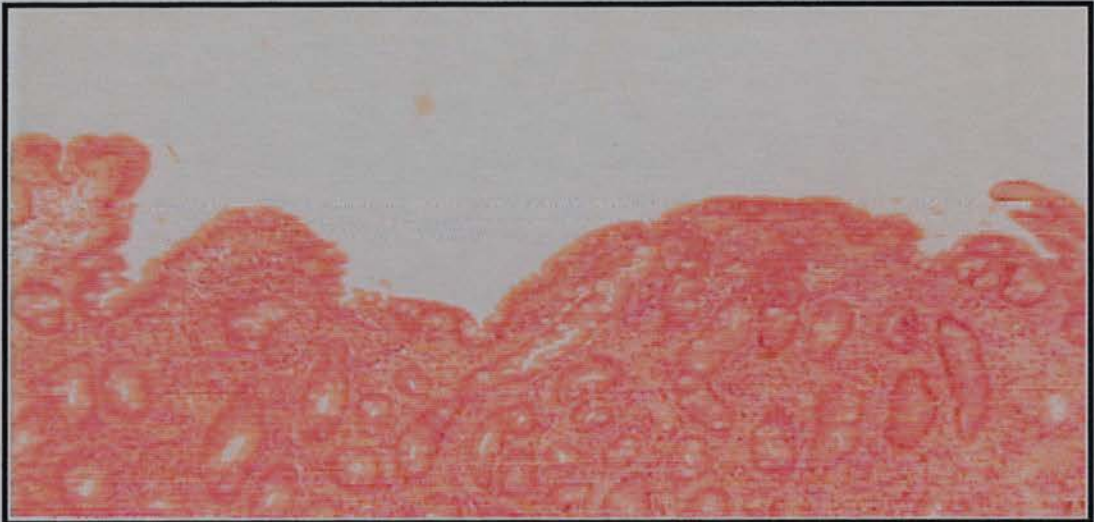


Figure 6.1b Duodenal biopsy of an IgA EmA negative coeliac disease patient

Another IgA EmA negative patient (patient 3 in **Table 6.4**) had initially presented with iron deficiency anaemia in 1993, when the diagnosis of CD was considered because of a raised serum and jejunal aspirate IgA AGA. Her endoscopic duodenal biopsies however, were reported as showing “non-specific changes” only, and a subsequent jejunal biopsy failed to obtain sufficient tissue. The diagnosis of CD was finally made 72 months later (after several missed appointments) on repeat duodenal biopsy, which showed severe PVA.

The last patient (patient 7 in **Table 6.4**) had presented to another hospital 5 years ago with symptoms of diarrhoea and weight loss, and was also found to have osteoporosis and hypocalcaemia. Her IgA AGA and IgA EmA were both negative, and on this basis, despite the clinical findings, duodenal biopsies were not performed. She was referred to our unit for a second opinion because of progressive weight loss and duodenal biopsies obtained, 60 months after her initial presentation, showed STVA consistent with untreated CD. Repeat serological testing remained negative. Repeat duodenal biopsies showed a good histological response to treatment with a GFD.

Other investigations, in search for a GI cause other than CD, were frequently performed in the IgA EmA negative patients. Eight patients (7 F: 1 male, age range 29 - 77 years, median 50 years) had colonic investigations (4 colonoscopies, 3 barium enemas and 2 flexible sigmoidoscopies). All of these examinations, including biopsies, were entirely normal except in 3 female patients. A 71 year old had diverticular disease, a 63 year old had proctitis due to collagenous colitis, and a 40 year old had ileal atrophy which prompted duodenal biopsy. Small bowel contrast studies were performed in 4 patients (3 F: 1male; age range 40 -77 years, median 56 years). Features of malabsorption were present in 2 patients, but the other 2 examinations were entirely normal.

6.4.2 HLA-DQ2 Status Compared to Serology & Clinical Features of Coeliac Disease Patients

Of the 43 blood samples obtained from the CD patients for DQ2 typing, the dot blots could be confidently stated to be positive or negative in only 36 samples. The DQ2 was positive in 22 patients (61%) and negative in 14 patients (39%). A typical example of dot blot template showing positive and negative results is shown in **Figure 6.2**.

Using the χ^2 test with Yates correction, this showed that DQ2 positive patients did not have a significantly higher frequency of positive serum IgA EmA results than DQ2 negative subjects ($\chi^2=2.72$, $0.5>p>0.1$) **Table 6.2**. The number of DQ2 positive patients with a raised IgA anti-tTG antibody concentration was not higher than in the DQ2 negative patients (χ^2 test: $p=0.17$). There was no significant difference in the IgA anti-tTG antibody concentration between these two groups (Mann-Whitney test: $p=0.17$). In addition, there was no significant difference in the clinical manifestations of CD in the DQ2 positive and negative untreated CD patients (χ^2 test: $p=0.85$). The DQ2 was determined in 12/13 IgA EmA negative CD patients. The result was positive in 4, negative in 7 and equivocal in 1 patient.

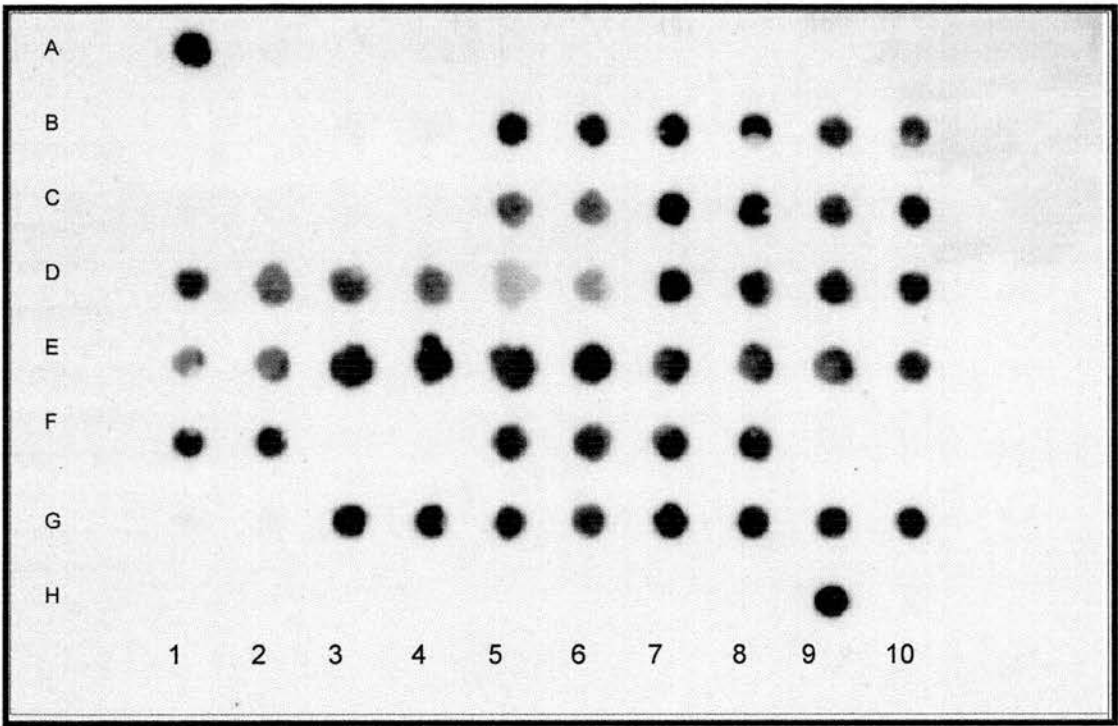


Figure 6.2 A dot blot template of HLA DQ2 results in CD patients. A1 & H9 were the positive controls, and A2 & H10 were the negative controls. B1 & B2 represented the PCR blanks. All other positions represented patient samples in duplicate horizontally. This photograph was obtained with permission from Kathleen Kingstone.

Table 6.2: HLA-DQ2 Status Compared to Serology & Clinical Features of CD

Parameter	HLA-DQ2 Positive Patients (n=22)	HLA-DQ2 Negative Patients (n=14)
Raised IgA Anti-tTG (>2950 units/ml)	16	7
IgA EmA Positive	18	7
Diarrhoea	17	10
Weight loss	13	11
Anaemia	12	7
Abdominal Pain	12	6

6.4.3 Serological Results in the Coeliac Disease & Control Patients

Of the 53 prospectively diagnosed CD patients studied, the serum IgA anti-tTG antibodies, IgA EmA, IgA AGA and IgG AGA were raised in 35 (66%), 40 (75%), 34 (64%) and 44 (83%) patients respectively. If the EmA negative patients had been excluded, the sensitivity of the IgA anti-tTG antibodies would improve to 80% (32/40). A sugar test was performed by 48 of the 53 patients. The L/R ratio was raised in 43 (90%) of these patients.

Of the 65 non-CD control patients with normal duodenal histology, the IgA anti-tTG, IgA AGA, and IgG AGA antibodies were positive in 2 (3%), 9 (14%), and 14 (22%) of these patients respectively. The serum IgA anti-tTG antibody concentrations in IgA EmA negative and positive CD patients, as well as controls are shown in **Figure 6.3**.

The sensitivities, specificities, positive and negative predictive values of the serological tests are compared in **Table 6.3**.

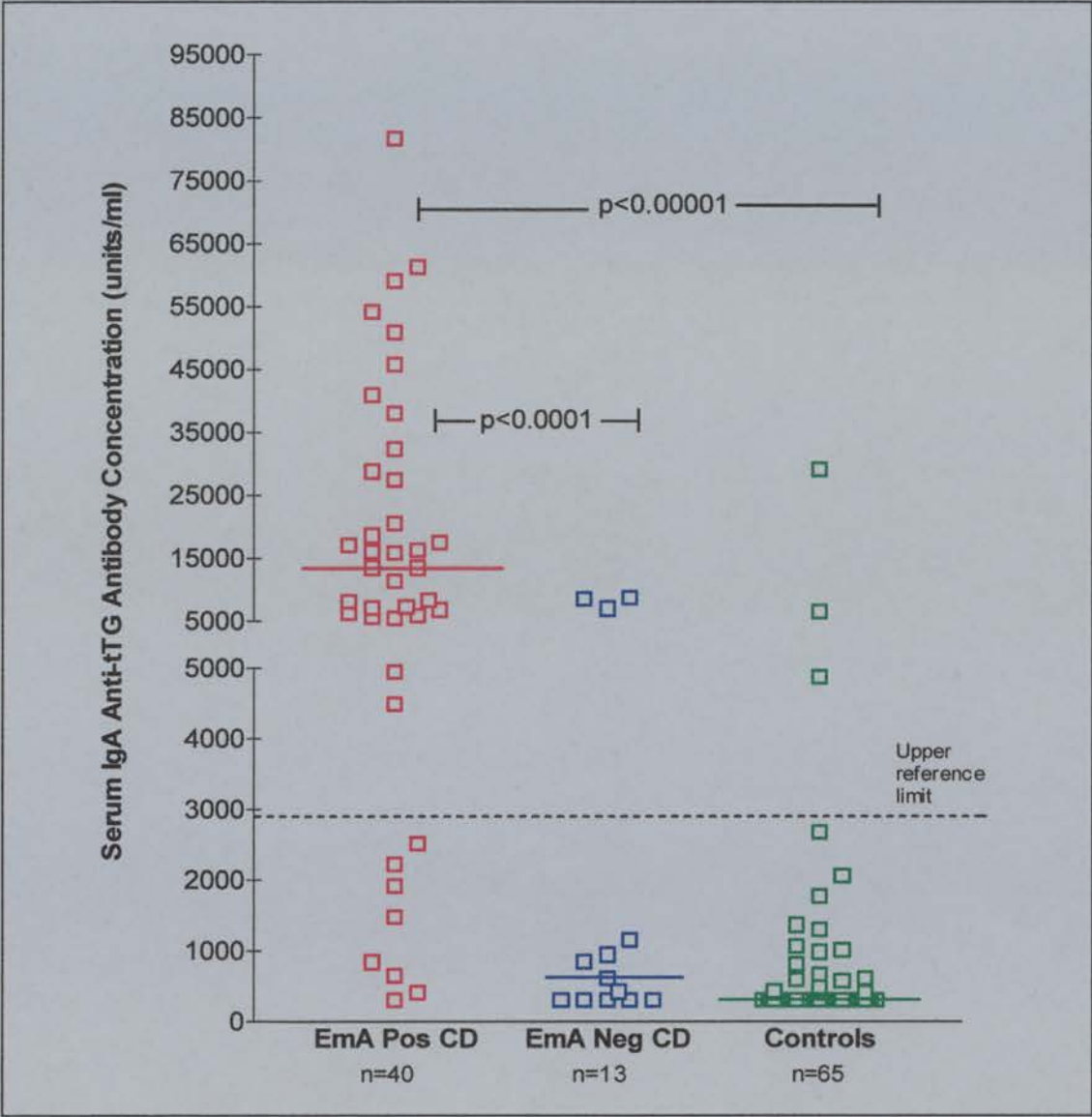


Figure 6.3: Serum IgA anti-tTG antibody concentrations in IgA EmA positive & negative CD patients, & disease controls. The horizontal lines indicate the median values.

Table 6.3: Performance of Serological Tests

Serological Test	Sensitivity (n=53)	Specificity (n=65)	Positive Predictive Value	Negative Predictive Value
IgA Anti-tTG	35 (66%)	63 (97%)	35/37 (95%)	63/81 (78%)
IgA EmA	40 (75%)	65 (100%)	40/40 (100%)	65/78 (83%)
IgA AGA	34 (64%)	56 (86%)	34/43 (79%)	56/75 (75%)
IgG AGA	44 (83%)	51 (78%)	44/58 (76%)	51/60 (85%)

6.4.4 Frequency Of IgA EmA Negative Coeliac Disease

Of the 53 untreated CD patients, the IgA EmA was negative in 12 patients and equivocal in 1 patient (25%). The demographic details, serological results and L/R ratios of these patients are shown in **Table 6.4**. Patient 8 was the only patient with an equivocal IgA EmA result. None of these patients had SIgAD although patients 1 and 9 both had low total serum IgA concentrations of 0.99 g/l and 0.78 g/l respectively.

Table 6.4: Results of IgA EmA Negative Coeliac Disease Patients

No.	Age/ Sex	IgA AGA (units/ml)	IgG AGA (units/ml)	IgA anti- tTG (units/ml)	L/R Ratio	Initial Duodenal Biopsy	Repeat Duodenal Biopsy	Duration of GFD (months)
1	29 F	11	206	300	0.042	PVA	Raised IELs	14
2	71 M	53	243	8440	0.084	PVA	Normal	19
3*	43 F	17	181	830	0.065	Severe PVA	Severe PVA	20
4	52 F	23	75	300	0.32	STVA	Mild PVA	5
5+	40 F	7	4	300	0.077	STVA	ND	16
6*	77 F	29	146	410	0.109	PVA	PVA	14
7+	52 F	5	8	940	0.028	PVA	Normal	4
8	70 F	589	315	6880	0.21	STVA	ND	4
9	71 F	6	236	605	0.094	STVA	Mild PVA	4
10	63 F	13	97	300	ND	PVA	Normal	10
11+	40 M	12	11	300	0.205	STVA	PVA	8
12*	37 F	46	86	8620	0.059	Severe PVA	Severe PVA	10
13	47 M	19	145	1150	0.009	PVA	Mild PVA	5

Abnormal serology and mucosal permeability are indicated in bold italics.

“ * ” indicates the patients without a histological improvement after gluten withdrawal.

“ + ” indicates the patients in whom all the serological markers were negative.

Patient 2 had DH, which prompted investigation by duodenal biopsies.

Patient 8 had an equivocal IgA EmA.

Patient 10 had collagenous colitis.

6.4.5 Other Non-invasive Methods for Detecting IgA EmA Negative Coeliac Disease Patients

Serum IgA anti-tTG antibody concentrations were raised in 3 EmA negative patients, one of whom had DH. These 3 patients also had raised IgA and IgG AGA concentrations. Of the 13 IgA EmA negative CD patients, the IgA AGA and IgG AGA concentrations were raised in 3 and 10 patients respectively. IgA/G/M-class ARA was negative in all of the IgA EmA negative patients. The L/R ratio was raised in 10/12 (83%) IgA EmA negative CD patients.

6.4.6 Repeat Duodenal Biopsy Histology in IgA EmA Negative Coeliac Disease Patients

Repeat small intestinal biopsy, following treatment with a GFD, was performed after a median duration of 10 months (range 4 - 20 months) in 11/13 IgA EmA negative CD patients. Duodenal histology had improved in 8 patients, but remained unchanged in 3 patients indicated by the symbol “*” in **Table 6.4**. Two of these 3 patients (patients 3 and 12) were non-compliant with their diet. The repeat serum IgA AGA concentration following treatment remained normal in patient 3, but increased from 46 units/ml to 70 units/ml in patient 12. These two patients had been re-biopsied after intervals of 20 and 10 months respectively. The other patient without a histological improvement had been compliant with therapy and her IgA AGA remained within normal limits. Her biopsies had been repeated after 14 months of dietary therapy.

Repeat duodenal biopsies were not obtained in 2 patients (patients 5 and 8 in **Table 6.4**). Patient 5 failed to attend for multiple appointments, and the procedure was technically difficult in patient 8. In addition, the duodenal histology had markedly improved in 2 (patients 7 and 11) of the 3 CD patients in whom all serological tests were negative. The other patient (patient 5) was the individual who failed to attend for

repeat biopsies. Although a histological remission was not demonstrated in all of the IgA AEM negative CD patients, they all reported a symptomatic improvement following gluten exclusion when reviewed at the coeliac clinic.

6.5 Discussion

This study showed that 25% of consecutively diagnosed CD patients do not express IgA EmA antibodies while consuming a normal gluten-containing diet. The IgA EmA status did not influence the clinical presentations, frequency of auto-immune diseases, duration of symptoms or calcium concentrations, but the serum albumin concentrations were significantly lower in EmA negative, than positive patients. Serum albumin levels have been found to be significantly lower in patients with refractory CD than in responsive CD patients (Stuart and Gent, 1998). As all of our IgA EmA negative CD patients responded clinically to gluten withdrawal, they do not appear to have refractory sprue, a condition in which there is little or no benefit from treatment with a GFD. In support of the diagnosis of CD in all of the EmA negative CD, was the symptomatic improvement following gluten withdrawal, and the histological improvement documented in 8 of the 11 patients who underwent repeat duodenal biopsy. In 2/3 patients in whom there was no histological response to gluten restriction, there was a clear history of dietary non-compliance. In support of this, the serum IgA AGA concentration was raised in one of these 3 subjects. Others have recently found that EmA negative CD patients who were compliant with their GFD all had a clinical response, although only 67% of patients had a histological improvement after 12 months of therapy on repeat biopsy (Dickey et al. 2000).

Many of the EmA negative patients had other radiological or endoscopic tests that would not have been performed if the diagnosis of CD had been suspected. These tests did not reveal any other causes of small intestinal mucosal inflammation with which the

histological findings could have been confused. In 2 of the EmA negative, duodenal biopsies were performed because of a pre-existing untreated condition which was known to be associated with CD - one patient had DH and the other had collagenous colitis. This latter patient required steroid treatment to control her symptoms, but was not on steroid treatment at the time of serum or duodenal biopsy sampling. It is well recognised that the sensitivity of IgA-class antibody tests is reduced by unrecognised SIgAD and immunosuppressive therapy which may produce false negative results (Rittmeyer and Rhoads, 1996), however none of our other newly diagnosed CD patients were receiving immune modifying drugs and or had SIgAD.

The HLA-DQ2 status ($\alpha 1^*0501$, $\beta 1^*0201$) could not be determined in all of the samples analysed due to difficulties in interpreting the staining pattern. These equivocal samples were excluded from the analysis. In the remaining CD patients, the $\alpha 1^*0501$, $\beta 1^*0201$ heterodimer was present in 22/36 (61%) subjects. This was lower than anticipated from the published work where this heterodimer has been estimated to be present in about 90-95% of British CD subjects (Sollid and Thorsby, 1993; Marsh et al. 1993). Those CD patients not expressing this heterodimer are known to almost always express the other CD-associated genes, HLA-DQ8 (DQ $\beta 1^*0302$) and HLA-DR4 (Marsh et al. 1993; Pena et al. 1998; Balas et al. 1997). As these genotypes were not investigated in this study, it was not possible to determine if the DQ2 negative patients in this study expressed these other associated genes.

Of the IgA EmA negative CD patients, the DQ2 was positive in 4/13 (31%) of patients. The frequency of DQ2 positive patients with a raised serum IgA anti-tTG antibody concentration or a positive IgA EmA was not significantly higher than in the DQ2 negative patients. In addition, there was no association between DQ2 status with clinical manifestations of untreated CD. Others have found no correlation between HLA genotype with specific clinical manifestations of CD (Greco et al. 1998). It is

likely that the aetiology of CD is multifactorial requiring the combination of environmental factors, the age of gluten introduction and genetic predisposition (both HLA and non-HLA genes), to modulate the clinical expression of the disease.

The sensitivity and specificity of IgA EmA and IgA anti-tTG antibodies in this study were 75% and 100%, and 66% and 97% respectively. These findings are comparable with other prospective studies that also used clinical indications for biopsy, and found a low IgA EmA sensitivity of 74-78% (Valdimarsson et al. 1996; Rostami et al. 1999a; Dickey et al. 2000), and IgA anti-tTG antibody sensitivity of 66% (Rostami et al. 1998). Indeed few studies have been able to reproduce the reported 100% sensitivity and specificity of IgA EmA (Kumar et al. 1989; Whelan et al. 1996; Ferreira et al. 1992). Rostami *et al.* have found that serum antibody positivity correlated with the severity of the mucosal lesion, such that the sensitivity of IgA EmA was 100% (17/17) in patients with TVA, but reduced to 31% (9/29) in those with PVA (Rostami et al. 1999b). This trend was also observed for IgA AGA (Rostami et al. 1999b).

Recent reports on the sensitivity and specificity of the IgA anti-tTG antibodies (also using the guinea pig liver source) have ranged from 85-98% and 90-98% (Sulkanen et al. 1998b; Dieterich et al. 1998; Lock et al. 1999b; Troncone et al. 1999; Biagi et al. 1999a). Only a small proportion of the untreated CD patients investigated in these studies, however, was IgA EmA negative: 10/136 (Sulkanen et al. 1998b), 1/106 (Dieterich et al. 1998), 1/27 (Lock et al. 1999b), and 0/39 (Biagi et al. 1999a). The lower sensitivity of IgA anti-tTG antibodies found in this study was partly due to the large number of IgA EmA negative CD patients included. If these EmA negative patients had been excluded, the sensitivity of IgA anti-tTG antibodies would improve to 80% (32/40).

The use of additional serological tests may help to identify some of these IgA EmA negative CD patients, as we detected 3 of these patients by measuring either the IgA anti-tTG antibodies or IgA AGA. ARA was of no additional value in screening as it did not detect any of these patients. IgG AGA detected 10 IgA EmA negative CD patients, but was also positive in 14/65 (22%) control patients, compared to 9/65 (14%) for IgA AGA. Although IgG AGA increased detection of IgA EmA negative CD patients, it may not be suitable for use as a screening tool as it is less specific than the IgA AGA. Combining IgG AGA with the more specific IgA AGA test may be the most appropriate compromise.

Demonstration of a characteristic mucosal lesion, coupled with the disappearance of clinical symptoms, signs and serological markers, remain the diagnostic standard for the diagnosis of CD. The documentation of a histological remission following gluten withdrawal is not done routinely in all centres. The clinical response to treatment often occurs rapidly, before there is a histological improvement (Howdle and Losowsky, 1992), which may still be incomplete after 2-4 years of gluten restriction (Grefte et al. 1988). A recent study (Selby et al. 1999), has compared duodenal biopsy histology with dietary gluten intake in 89 adult CD patients who were established on a GFD for a duration of 8.3 ± 6.7 years. They found persistent villous atrophy in 38 (43%) patients, up to 8 years after the initiation of dietary therapy, and this was not related to the ingestion of trace amounts of gluten. Others have found that small amount of gluten may be tolerated in CD without initiating a significant AGA response or gross morphological change (Montgomery et al. 1988).

The presence of a symptomatic, but not histological improvement in 3 of our patients, after a period of up to 20 months therapy with a GFD, therefore does not exclude the diagnosis of CD in these subjects. As the mucosal lesions in CD are more pronounced in the proximal small bowel than they are distally, and the histological recovery also

proceeds from the distal to the proximal small bowel (MacDonald et al. 1964), it is possible that the persistent abnormalities in these patients were confined to the duodenum.

It is recognised that gluten-sensitivity may exist without detectable serum antibodies (Mulder and Rostami, 1998). Indeed, in our study, the serological markers were all negative in 3 of the untreated CD patients (patients 5, 7 and 11 in **Table 6.4**). The L/R ratio was raised in 2 of these individuals. There was a symptomatic benefit in all 3 patients, and a histological improvement was documented in 2 of them following gluten withdrawal. The other patient (patient 5) failed to attend for repeat biopsies. This patient had evidence of ileal dysfunction with severe bile acid malabsorption, vitamin B₁₂ deficiency and weight loss, and was found to have ileal atrophy when her ileum was biopsied at colonoscopy.

Ileal function and morphology in CD have not been widely studied. It is likely that the distal small bowel is usually spared from damage because dietary gluten is almost completely digested in the proximal gut (Rolny et al. 1990). It has been shown however, that high doses of oral gluten can induce severe ileal villous atrophy (Rubin et al. 1962). It has also been recognised that CD can clinically manifest after gastric resection, presumably because of the resulting increased rate of gastric emptying into gut and resulting heavier gluten exposure (Rolny et al. 1990). Rolny *et al.* reported the finding of patchy ileal inflammation, but without villous atrophy, in 12 untreated CD patients in whom duodenal or jejunal biopsies showed either STVA or total villous atrophy (TVA) (Rolny et al. 1990). Serum vitamin B₁₂ concentrations were low in 3 of 9 patients, in whom the levels were measured before starting a GFD (Rolny et al. 1990). Others have found severe ileal villous atrophy in untreated CD patients (Silk et al. 1975), and this has also been demonstrated in a post-mortem study (Thomson,

1974). The distal small bowel was shown to have the ability to adapt to the damage and loss of absorptive capacity in the proximal gut (Silk et al. 1975).

This study shows that “false-negative” IgA EmA CD may be more common than previously recognised. The inclusion of these patients in CD studies is important as the reporting of predominantly EmA positive patients, distorts the performance of serological tests. EmA negative patients are also a challenge to diagnosis and follow-up. The varying presentations of CD require a high degree of clinical suspicion and alertness to make an accurate diagnosis. The consequences of a delay in the diagnosis of CD are significant as there is an increased frequency of lymphoma in untreated CD in the order of 40-100 fold (Holmes et al. 1989; Leonard et al. 1983), and also of malignancies at other sites (Swinson et al. 1983). The high prevalence of IgA EmA negative patients in these consecutively diagnosed CD patients shows the importance of maintaining intestinal biopsy as the “gold standard” for the diagnosis of CD. Small intestinal biopsy should be performed in seronegative patients with a clinical suspicion of CD and should be repeated following treatment with a GFD.

The absence of a humoral response to tTG or to EmA in some of our untreated CD patients casts doubt on the role of autoantibodies in the pathogenesis of CD. The observations that CD is common in SIgAD (Collin et al. 1992; Cataldo et al. 1999), and may occur in severe hypo-gammaglobulinaemia (Webster et al. 1981), also make it unlikely that antibodies contribute directly to the mucosal atrophy. It is possible that immunologic responses to other as yet unidentified autoantigens may underlie the mucosal inflammation seen in the EmA negative patients. The relationship between serological markers with small intestinal histology will be explored in chapter 8.

CHAPTER 7: GUT MUCOSAL IMMUNITY TO TISSUE TRANSGLUTAMINASE IN COELIAC DISEASE & OTHER GASTROINTESTINAL DISORDERS

7.1 Introduction

In the previous two chapters, the systemic response to tTG was investigated in untreated CD patients, disease and healthy volunteers. As the mucosal and systemic immune systems are distinct in the many respects previously described, the nature of the mucosal immune response to tTG cannot be extrapolated from serum results and the gut must be studied directly. We have proposed that if there is a local production of anti-tTG antibodies in the GI submucosa, they should be secreted into the gut lumen and could therefore be measured in WGLF.

There is much evidence that intra-luminal IgA antibodies are locally produced. In support of this were the findings that the number of Ig producing cells in human jejunal mucosa are increased in untreated CD (IgA>IgM>IgG) (Colombel et al. 1990; Wood et al. 1987; Jonard et al. 1984), and raised levels of IgA and IgM AGA in jejunal fluid (O'Mahony et al. 1991; Lavo et al. 1992; Colombel et al. 1990), have been demonstrated in CD. In addition, a marked dissociation between antibody responses to gliadin in serum and gut secretions has previously been demonstrated in CD (O'Mahony et al. 1991). This means that the nature of the gut mucosal response to tTG cannot be inferred from the serum findings in the preceding chapters. The difficulties in encountered in obtaining suitable material to study mucosal immunity were outlined in **section 2.3.5.**

The WGL technique has been validated as a safe, non-invasive means of studying the intra-luminal secretion of gut-derived antibodies (O'Mahony et al. 1991; Brydon et al. 1993; O'Mahony et al. 1991). There is little variation in Ig content once the WGLF is clear (O'Mahony et al. 1990), and the addition of protease inhibitors protects against the loss of Igs due to proteolysis (Gaspari et al. 1988). IgA is the most abundant Ig in WGLF, and most of it is predominantly of secretory type (Gaspari et al. 1988), which is more resistant to proteolysis than the monomeric serum Igs (Brown et al. 1970). Normal reference ranges of IgG and albumin in WGLF have previously been established (Brydon et al. 1993; O'Mahony et al. 1990), and an elevated WGLF IgG >10 µg/ml has been shown to be indicative of active disease in IBD patients (Brydon et al. 1993; Choudari et al. 1993). Anti-gliadin antibodies have previously been measured in the WGLF of untreated CD patients (O'Mahony et al. 1991), but as yet, there are no studies on the presence of anti-tTG antibodies in WGLF. In the gut, tTG is predominantly submucosal (Patel et al. 1985), and its activity is increased in the jejunal mucosa of untreated CD patients (Bruce et al. 1985). As the enzyme is widely distributed throughout the length of the gut (Patel et al. 1985), it would not be surprising if locally produced anti-tTG antibodies were not specific for CD.

The aims of this chapter were to investigate the presence of IgA anti-tTG antibodies in the WGLF of untreated CD patients, as well as in disease and healthy controls, and to determine whether there was a correlation between the mucosal and systemic immune responses to tTG. The concentration of WGLF IgA anti-tTG antibodies is also related to disease activity in IBD patients. The source of intra-luminal IgA anti-tTG antibodies is speculated by comparison of these antibodies with matched WGLF IgG and albumin concentrations. WGLF IgG and albumin are both thought to be predominantly derived from plasma leakage into the gut lumen.

7.2 Patients

All untreated CD patients and disease controls recruited into this study had presented to the GI unit at the Western General Hospital in Edinburgh for investigation of abdominal symptoms or for further assessment of pre-existing GI diseases. A total of 300 samples from 286 subjects were analysed (**Table 7.1**).

Untreated CD Patients:

During the study period from 1997-1999, a total of 53 biopsy-proven untreated CD patients were prospectively diagnosed. All of these patients were asked to participate in the study by performing a WGL procedure for research purposes, except for the IDDM patients who were excluded because of the prolonged fasting required. Verbal consent was obtained from all consenting patients. Some of these patients however, because of the advanced age at presentation and bowel symptoms, were also having lower GI investigations requiring bowel cleansing. The WGLF was therefore obtained from these patients as part of their routine clinical assessment.

A total of 36 patients successfully completed the WGL procedure, a further 3 patients had failed to complete the WGL protocol and were excluded. The reason for failure to complete the test was usually nausea, bloating and vomiting. The remaining patients approached declined to participate. Paired WGLF and serum samples were prospectively obtained from these 36 CD patients (27 F: 9 M; age range 27-77 years, median 52 years) all of whom were on a normal gluten-containing diet at the time of serum sampling and undergoing the WGL procedure. Three of the 36 patients included also had DH.

Disease controls:

The method for selecting disease control samples was described in **section 5.2**. All of these patients had undergone a WGL procedure during 1996 as part of their routine

clinical investigations. A matched WGLF specimen was available for 251 of the 259 disease controls serum samples studied in chapter 4. These 251 WGLF samples were obtained from 237 patients. All WGLF samples had been stored at -70°C and had not previously been used.

Included in this group were 140 histologically confirmed IBD patients who included 64 Crohn's disease patients and 76 UC patients. The disease distribution and previous surgical procedures of these patients were recorded. Some of the Crohn's disease patients had mixed small and large bowel involvement, and/or had undergone surgical resection prior to their WGL procedure. However 30 patients (11 small bowel, 10 large bowel, 8 ileo-colonic and 1 oral) had not undergone previous surgical resection. One of these patients had isolated duodenal and jejunal involvement. Twenty-one UC patients had undergone restorative panprocto-colectomy and ileo-anal pouch formation prior to undergoing a WGL procedure and were being assessed for the presence of pouchitis.

Thirty-six patients with IBS were included and the WGLF IgG was <10 µg/ml in all of these subjects. Fifteen patients with idiopathic or infective diarrhoea were included. Four patients had a confirmed infective aetiology with the isolation of *Giardia* (2), *Salmonella* (1) and *Campylobacter* (1).

The diagnoses of the 46 patients in the miscellaneous group included diverticular disease, GI malignancies, colonic polyps, radiation enteritis, ischaemic colitis, alcoholic liver disease, anaemia of unknown aetiology, bile acid malabsorption and also patients undergoing colonoscopy because of a family history of colonic malignancy. Rare diagnoses in this group included patients with rheumatoid disease with amyloidosis on duodenal biopsy (n=1), Cronkhite-Canada syndrome (n=1), pneumatosis cystoides intestinalis (n=1), and ileo-caecal carcinoid (n=1).

Healthy volunteers:

Paired serum and WGLF samples were obtained from 13 consenting healthy volunteers between 1998 and 1999. These asymptomatic individuals were participating as healthy controls in an on-going *E. Coli* research project. Small intestinal biopsies were not performed in any of these individuals.

Table 7.1: Disease Categories & Patient Demographics

Disease Category	Number of Samples	Number of Patients	Gender (F:M)	Age Range (years)	Median Age (years)
<i>Untreated CD</i>	36	36	27:9	27-77	52
UC	78	76	33:43	15-80	45
Crohn's disease	76	64	37:27	17-82	41
IBS	36	36	25:11	16-91	39
Infective/Idiopathic Diarrhoea	15	15	9:6	22-78	51
Other Diagnoses	46	46	28:18	19-82	54
Healthy Volunteers	13	13	10:3	23-52	35
TOTAL	300	286			

Number of disease control samples = 251

Number of disease control patients = 237

7.3 Methods & Statistical Analysis

Tests Performed on WGLF Samples:

The WGL procedure, specimen collection and processing have been previously described in **sections 4.1.2** and **4.1.3** respectively. WGLF IgA anti-tTG antibodies, IgA AGA, total IgG and albumin were measured in all WGLF samples using the methods described in **sections 3.3, 4.2.3, 4.2.6** and **4.3** respectively. The use of a serum reference standard in the method for the measurement of WGLF IgA anti-tTG was validated as previously described in **section 3.2.2**. The upper reference limit of IgA anti-tTG in WGLF was 110 units/ml and was determined as previously described in **section 3.1.6**. The upper reference limit of WGLF IgA AGA was 5 units/ml as previously described in **section 4.2.3.2**. The previously established normal reference ranges for WGLF IgG and albumin of $<10\text{ }\mu\text{g/ml}$ and $<26\text{ }\mu\text{g/ml}$ respectively were used (Brydon et al. 1993).

IgA anti-tTG antibodies were also measured in the processing solution as new-born calf serum was used during the WGLF processing. In untreated CD patients, the WGLF total IgA was measured as previously described in **section 4.2.5**. IgA EmA was measured in the WGLF samples of all untreated CD patients, healthy volunteers, and the disease controls with an elevated IgA anti-tTG antibody concentration as previously described in **section 4.4.3**.

Serum Samples:

IgA anti-tTG antibodies, IgA AGA and IgA EmA were measured in all serum samples using the methods described in **section 3.3, 4.2.3** and **4.4.3**. The serum albumin, the total IgA and total IgG concentrations in serum were measured in the untreated CD

patients using the routine clinical chemistry laboratory service. The reference ranges for serum albumin and total IgA were 35-55 g/l and 1.01-4.23 g/l respectively.

The statistical methods applied were described in **section 4.9**. The WGLF IgA anti-tTG antibody and IgA AGA concentrations in untreated CD patients, disease and healthy controls were compared using the Mann-Whitney test. The WGLF IgA anti-tTG antibody concentrations in IgA EmA positive and negative WGLF specimens were also compared using the Mann-Whitney test. The median concentrations of IgA anti-tTG in the EmA positive and negative WGLF samples were also determined. The χ^2 test was used to compare the number of IBD and untreated CD patients with a raised WGLF total IgG. This comparison was also made between the IBD patients and the IBS subjects/healthy volunteers. Pearson's correlation was applied to determine the correlation between WGLF IgA anti-tTG antibody concentrations and WGLF total IgA, and the significance was determined by linear regression. ROC curves were plotted to allow the comparison of the performance of the IgA anti-tTG antibody ELISA in WGLF and serum across a wide range of concentrations.

7.4 Results

7.4.1 WGLF IgA Anti-tTG Antibodies & IgA EmA in Coeliac Disease Patients

The IgA anti-tTG antibody concentration was raised in 30/36 (83%) of untreated CD WGLF samples (**Table 7.2**). The WGLF IgA EmA was positive in 25 of these 30 patients. The WGLF IgA anti-tTG antibody concentrations were significantly higher in the untreated CD patients than in any of the disease controls or the healthy volunteers (Mann-Whitney test, $p < 0.0001$) (**Figure 7.1**). Two female patients, ages 29 and 71 years, had low total serum IgA concentrations of 0.99 g/l and 0.78 g/l respectively (reference range 1.01-4.23 g/l), but none of the CD patients had SIgAD (< 0.07 g/l).

In the untreated CD patients, 24/36 (67%) WGLF samples were IgA EmA positive. The IgA anti-tTG antibody concentrations were raised in all of these samples. Of the remaining WGLF samples, 11 were IgA EmA negative and 1 was equivocal. The patient with an equivocal WGLF IgA EmA and 5 of the IgA EmA negative WGLF samples had raised IgA anti-tTG antibody concentrations. The IgA anti-tTG antibody concentrations were significantly higher in the IgA EmA positive (median 1090 units/ml, range 155-4770 units/ml) than EmA negative (median 75 units/ml, range 15-1680 units/ml) WGLF samples (Mann-Whitney test, $p < 0.0002$) (**Figure 7.2**). The concordance rates between WGLF and serum IgA anti-tTG antibodies and IgA EmA were 83% and 86% respectively.

Table 7.2: WGLF Results in Coeliac Disease & Control Patients

Disease Category	WGLF IgA tTG Median (Range) units/ml	Number with WGLF IgA tTG > 110 units/ml	WGLF IgA AGA Median (Range) units/ml	Number with WGLF IgG ≥10 µg/ml
<i>Untreated CD</i> (n=36)	530 (15-4770)	30 (83%)	9.2 (0.1-380.0)	4 (11%)
UC (n=78)	15 (15-8480)	3 (4%)	0.4 (0.1-57.0)	25 (32%)
Crohn's Disease (n=76)	15 (15-135)	1 (1%)	0.5 (0.1-29.5)	38 (50%)
IBS (n=36)	15 (15-200)	1 (3%)	0.4 (0.1-5.6)	0
Infective/Idiopathic Diarrhoea (n=15)	15 (15-180)	2 (13%)	0.4 (0.2-18.7)	0
Other Diagnoses (n=46)	15 (15-180)	2 (4%)	0.3 (0.1-17.1)	3 (7%)
Healthy Volunteers (n=13)	15 (15-145)	1 (8%)	0.3 (0.2-10.0)	0

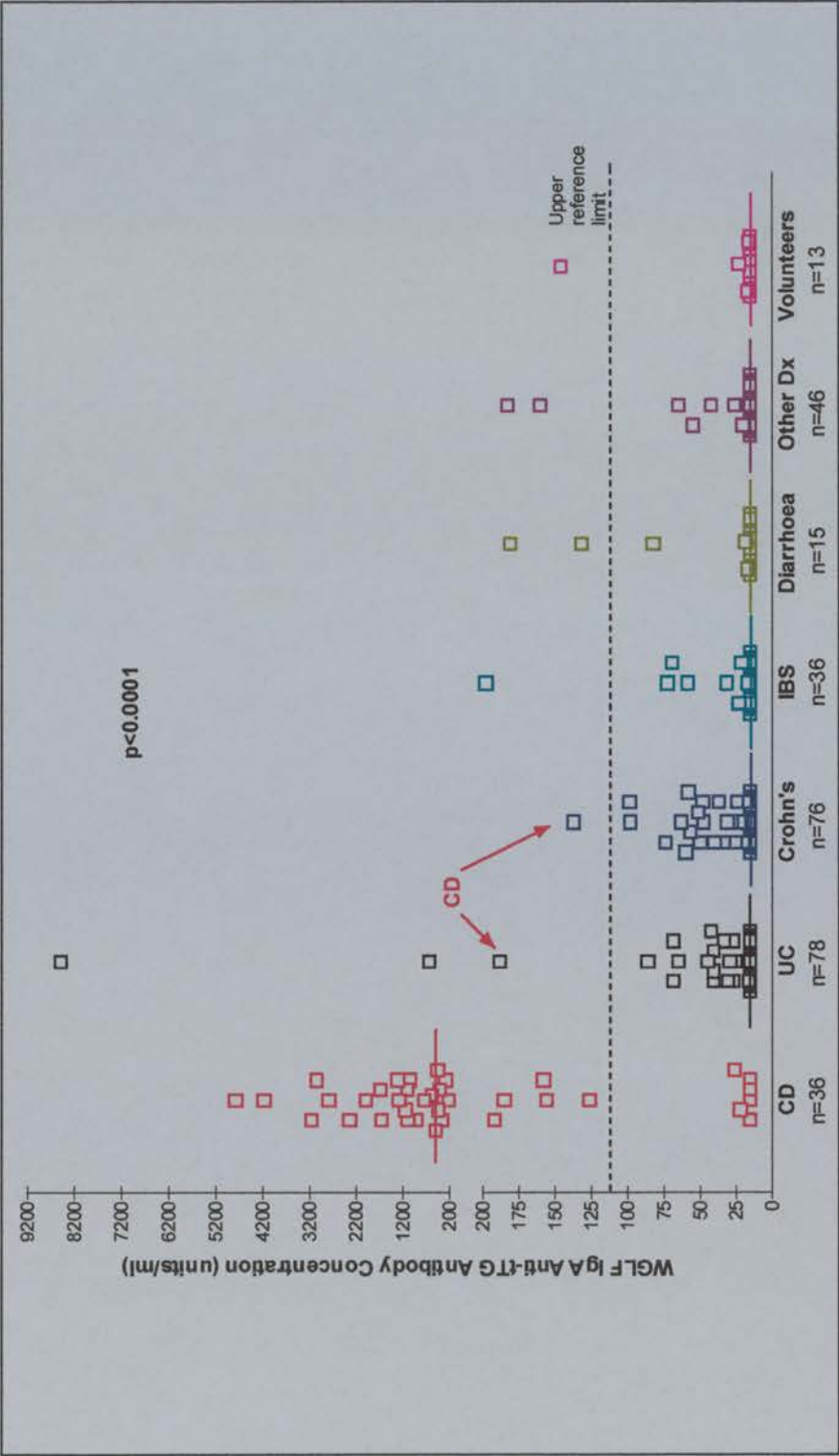


Figure 7.1: WGLF IgA anti-tTG antibody concentrations in CD patients, disease controls & healthy volunteers. The horizontal lines indicate the median values. The red arrows indicate the two disease control patients with CD.

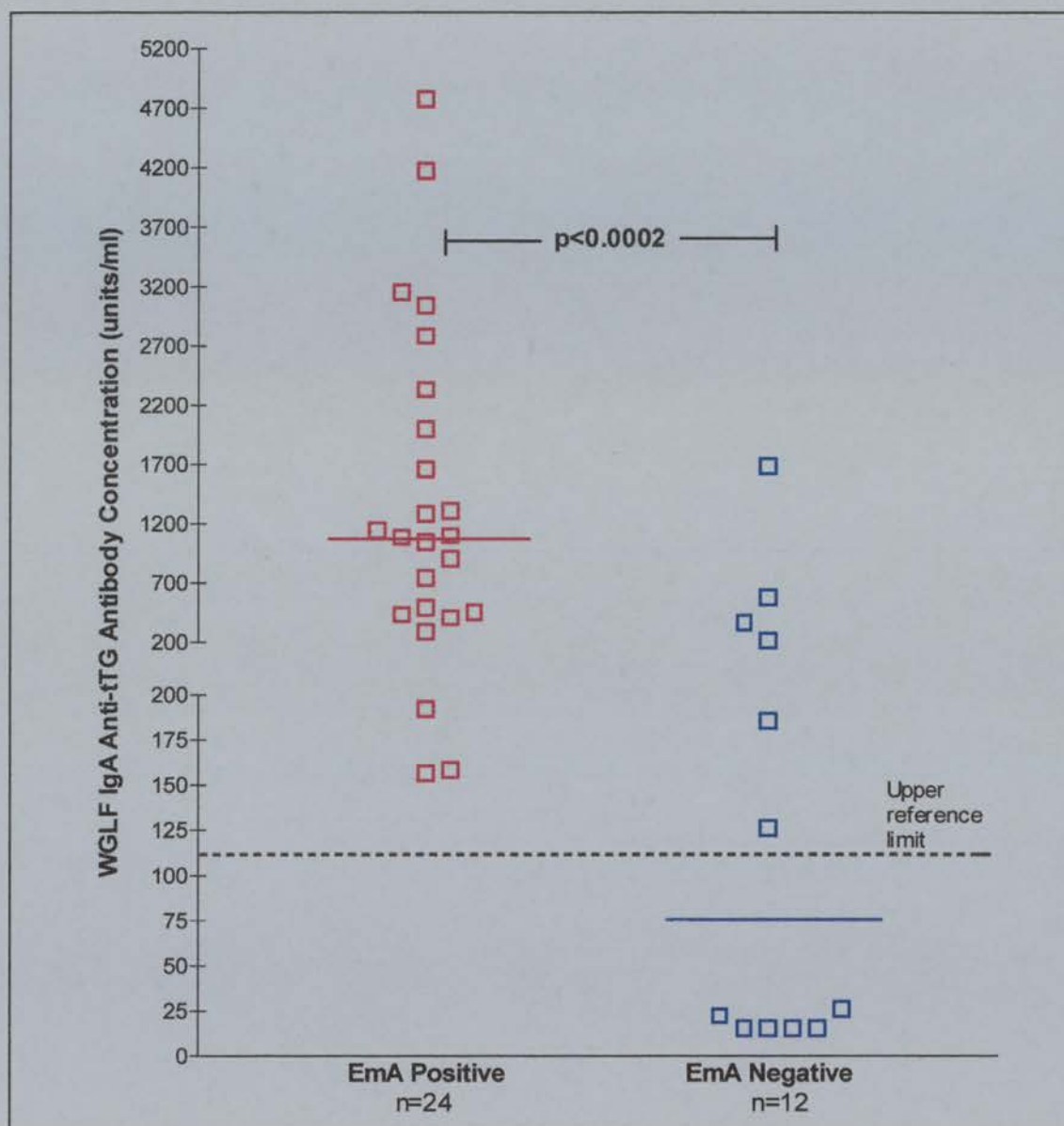


Figure 7.2: WGLF IgA anti-tTG antibody concentrations in WGLF IgA EmA positive & negative CD patients. The horizontal lines indicate the median values. The WGLF IgA anti-tTG antibody concentrations are significantly higher in the EmA positive than the EmA negative CD patients (Mann-Whitney test: $p<0.0002$).

7.4.2 WGLF IgA Anti-tTG Antibodies & IgA EmA in Disease Controls

The WGLF IgA anti-tTG antibody concentrations were raised in 9/251 (4%) of all disease control samples (**Table 7.2**). The IgA EmA staining pattern was identical for serum and WGLF samples. The WGLF IgA EmA was negative in all of the disease controls with a raised WGLF IgA anti-tTG antibody concentration. A typical positive WGLF sample is shown in **Figure 7.3**.

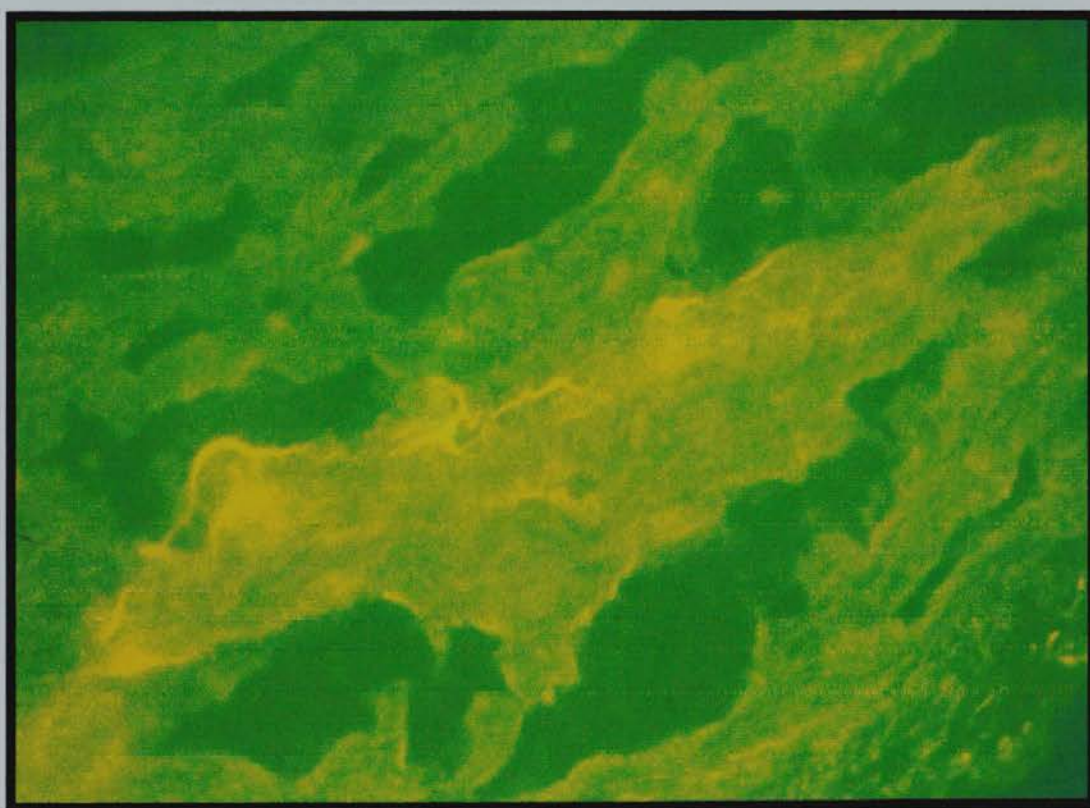


Figure 7.3: A positive WGLF IgA EmA in an untreated CD patient. A honeycomb immunofluorescence pattern is seen.

Ulcerative colitis patients:

Three patients with inactive ulcerative colitis (WGLF IgG <10 µg/ml), had raised WGLF IgA anti-tTG antibody concentrations. The first subject, with a WGLF IgA anti-tTG concentration of 8480 units/ml, was a 67 year old female patient with a known proctocolitis who was being investigated for recent weight loss without alteration of her bowel habit. Colonoscopy showed a quiescent colitis, which was in keeping with her normal WGLF IgG and albumin concentrations. Endoscopic duodenal biopsies were normal. The second patient with a WGLF IgA anti-tTG antibody concentration of 625 units/ml, was an asymptomatic 60 year old man with recto-sigmoid colitis and a previous tubular adenoma. A WGL was performed as bowel preparation prior to a surveillance colonoscopy. No further polyps were identified and duodenal biopsies were not clinically indicated. The matched serum IgA anti-tTG antibodies, IgA EmA and IgA AGA were normal in both of these patients.

The third patient with a WGLF IgA anti-tTG of 185 units/ml, was a 51 year old man with an ulcerative pancolitis and sclerosing cholangitis, who presented with diarrhoea, weight loss, anaemia, and folate deficiency. Duodenal biopsies obtained showed severe PVA consistent with CD. Repeat duodenal biopsies 5 months after treatment with a GFD showed entirely normal villous architecture. Of interest, his serum IgA anti-tTG antibody concentration was raised at 18100 units/ml, but the IgA EmA and IgA AGA were negative. Interestingly, this was the only one of the 3 UC patients who had an elevated WGLF IgA AGA with a concentration of 6.3 units/ml. The WGLF IgA EmA was negative in all of these 3 samples. All other UC patients had a WGLF IgA anti-tTG antibody concentration of < 85 units/ml.

Crohn's disease patients:

Only 1 Crohn's disease patient had an elevated WGLF IgA anti-tTG with a concentration of 135 units/ml. This was a 66 year old lady who initially presented with profuse watery diarrhoea, weight loss and a low vitamin B₁₂ level secondary to pernicious anaemia. She was also found to have diverticular disease at colonoscopy and pancreatic exocrine insufficiency. Duodenal biopsies showed STVA consistent with CD. She responded to treatment with a strict GFD, but re-presented one year later with watery diarrhoea, weight loss and a low serum albumin of 33 g/l. Repeat duodenal biopsies and a small bowel follow-through were all normal, but a sugar permeability test was grossly elevated at 0.24. There was no evidence of bacterial colonisation. A WGL was performed as bowel preparation prior to a barium enema examination, which was normal. The WGLF IgA EmA and AGA were negative as were the matched serum IgA anti-tTG, IgA EmA and IgA AGA. Unfortunately, serology was not documented prior to therapy with a GFD to allow comparison. She responded to oral corticosteroid therapy and has been managed for small bowel IBD although this has not been histologically confirmed.

The Crohn's disease patient with isolated duodenal and jejunal involvement, had active WGLF inflammatory markers (total IgG of 49 µg/l, albumin of 122 µg/l), a high WGLF IgA AGA concentration of 30 units/ml, but a normal WGLF IgA anti-tTG antibody concentration of 27 units/ml. The matched serum IgA AGA concentration was also elevated at 338 units/ml, while the serum IgA anti-tTG antibody concentration and the IgA EmA were negative. This patient had the highest WGLF and serum IgA AGA concentration of all the Crohn's disease patients and the highest serum IgA AGA concentration of all the IBD patients. All other Crohn's disease patients had a WGLF IgA anti-tTG antibody concentration of <100 units/ml.

The Crohn's disease patient with a raised serum IgA anti-tTG antibody concentration of 5260 units/ml, a positive serum IgA EmA and normal small intestinal biopsy discussed in **section 5.4.4**, had a normal WGLF IgA anti-tTG antibody concentration of 25 units/ml and a negative WGLF IgA EmA result.

Irritable bowel syndrome patients:

A 31 year old female patient with IBS and mild bile acid malabsorption, had a raised WGLF IgA anti-tTG antibody concentration of 200 units/ml. This patient had a normal small bowel follow through and colonic biopsies, but small intestinal biopsies were not obtained. All other IBS patients had WGLF IgA anti-tTG antibody concentrations of <75 units/ml.

Idiopathic and infective diarrhoea patients:

Two patients with idiopathic diarrhoea had raised WGLF IgA anti-tTG antibody concentrations. The first patient with a WGLF IgA anti-tTG antibody concentration of 130 units/ml, was a 39 year old insulin-dependent diabetic, with a history of alcohol excess and chronic diarrhoea. He had a normal upper endoscopy and duodenal biopsies, colonoscopy, small intestinal permeability and barium follow through examination. His pancreatic function tests were mildly abnormal, but an endoscopic retrograde cholangio-pancreatography showed no evidence of chronic pancreatitis, and he had no response to pancreatic enzyme supplements. A breath hydrogen test showed no evidence of bacterial overgrowth and he did not respond to empirical antibiotic therapy. The WGLF IgA AGA and IgA EmA were both normal. The serum IgA-class anti-tTG antibody, EmA and AGA were all within normal ranges.

The other patient with a WGLF IgA anti-tTG antibody concentration of 180 units/ml, was a 55 year old man with a complicated past medical history including a cardiac

transplant, chronic renal impairment, chronic obstructive airways disease and type II diabetes mellitus who presented with watery diarrhoea. Upper endoscopy with duodenal biopsies, as well as colonoscopy and biopsies were all normal. Aspirates from the duodenum and colon did not isolate any opportunistic or other organisms complicating his immunosuppressive therapy. The serum antibodies were all normal. All other patients in this group had a WGLF IgA anti-tTG antibody concentration of <80 units/ml.

Other diagnoses:

Two patients in this group had elevated WGLF IgA anti-tTG concentrations. The first patient with a WGLF IgA anti-tTG concentration of 180 units/ml was a 42 year old man presenting with alcoholic cirrhosis, chronic pancreatitis and type II diabetes mellitus. Serum IgA-class anti-tTG, EmA, and AGA antibodies were all negative, and jejunal biopsy and colonoscopy were normal. He responded to pancreatic enzyme supplementation. The other patient with a WGLF IgA anti-tTG concentration of 160 units/ml, was a 73 year old man with Cronkhite-Canada syndrome from whom small intestinal biopsies were not obtained. His serum IgA anti-tTG antibody and AGA concentrations were raised, but the IgA EmA was negative. All other patients in this group had a WGLF IgA anti-tTG antibody concentration of <65 units/ml.

7.4.3 WGLF IgA Anti-tTG Antibodies & IgA EmA in Healthy Controls

The only healthy volunteer with a raised WGLF IgA anti-tTG antibody concentration of 146 units/ml was a 23 year old dietetic student. The WGLF IgA AGA and EmA were both normal, and the matched serum antibodies were all within reference range. All other volunteers had WGLF IgA anti-tTG antibody concentrations of <23 units/ml.

7.4.4 WGLF IgA AGA in Coeliac Disease Patients, Disease & Healthy Controls

The WGLF IgA AGA concentrations were raised in 28/36 (78%) of CD patients and in 24/264 (9%) of the control patients. The WGLF IgA AGA concentrations were significantly higher in the untreated CD patients than in all other disease controls or healthy volunteers (Mann-Whitney test, $p < 0.0002$) (**Figure 7.4**). The WGLF IgA AGA and IgA anti-tTG antibody concentrations did not correlate significantly in the untreated CD patients ($r^2 = 0.017$, $p = 0.43$).

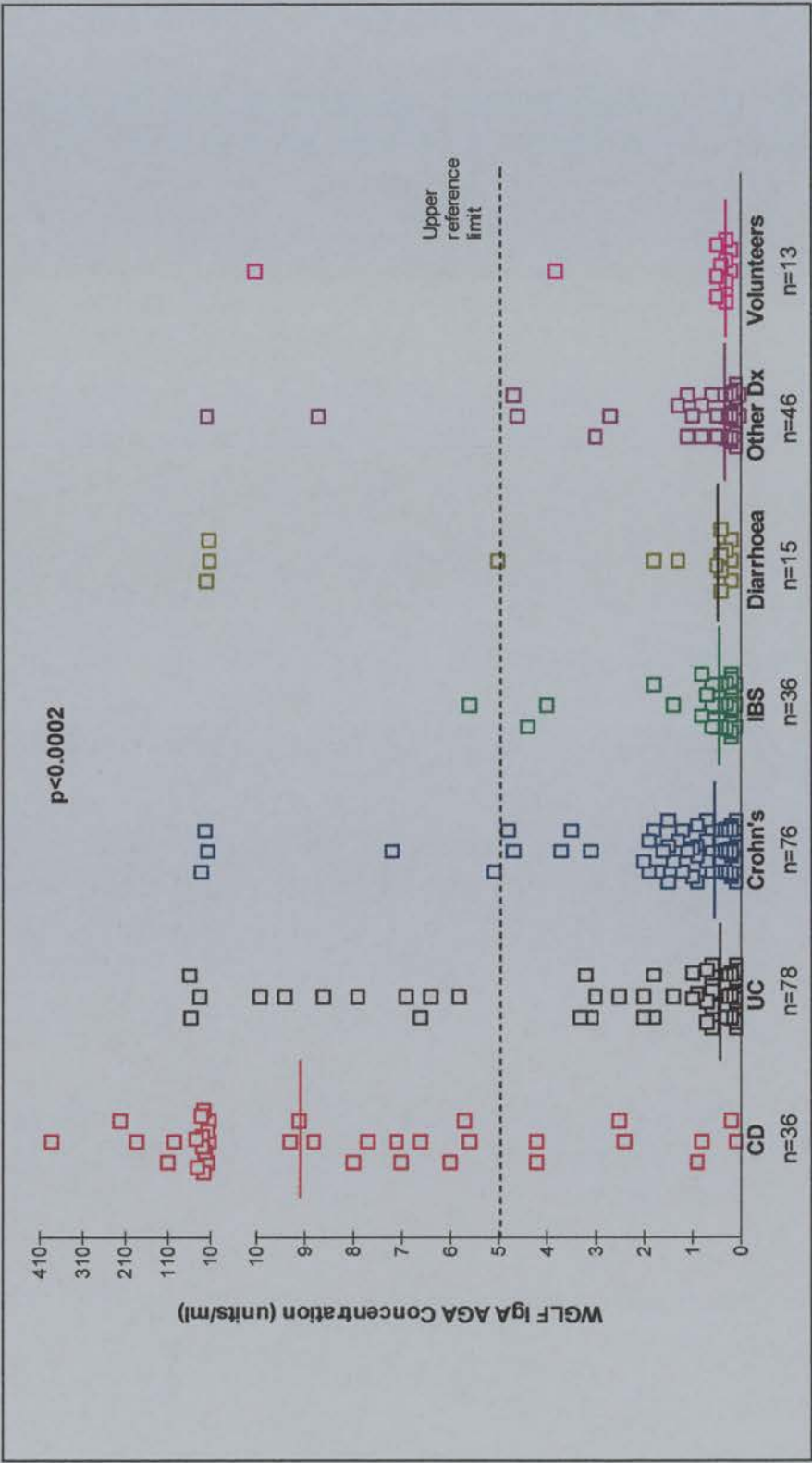


Figure 7.4: WGLF IgA AGA concentrations in CD patients, disease controls & healthy volunteers. The horizontal lines indicate the median values. The WGLF IgA AGA concentrations were significantly higher in the CD patients than the disease controls or healthy volunteers (Mann-Whitney test: $p<0.0002$).

7.4.5 Comparison of Paired WGLF & Serum Antibodies in Coeliac Disease Patients

WGLF & Serum IgA anti-tTG antibodies:

The WGLF and serum IgA anti-tTG antibody concentrations were raised in 30/36 (83%) and 26/36 (72%) of untreated CD patients respectively. The ROC curves showing the performance of the IgA anti-tTG assay in WGLF and serum are shown in **Figure 7.5a** and **7.5b** respectively. The graphs plot the assay sensitivity against 1-specificity for a number of different possible cut-off points. A high assay specificity is indicated if the curve follows the y-axis. The best cut-off point that balanced the “cost” of a false negative or false positive result was that which maximised the sensitivity and specificity. This point was the point nearest the top left-hand corner of the graph. These graphs demonstrate that the WGLF assay is more sensitive and specific than the assay in serum. The concordance rate for WGLF and serum IgA anti-tTG antibodies was 83%. In addition, in 5 CD patients the IgA anti-tTG antibody concentrations were raised in the WGLF but not the serum samples (**Table 7.3**). The small intestinal histology showed PVA in all of these 5 subjects. There was no correlation between WGLF and serum IgA anti-tTG antibody concentrations ($r^2 = 0.072$, $p = 0.11$).

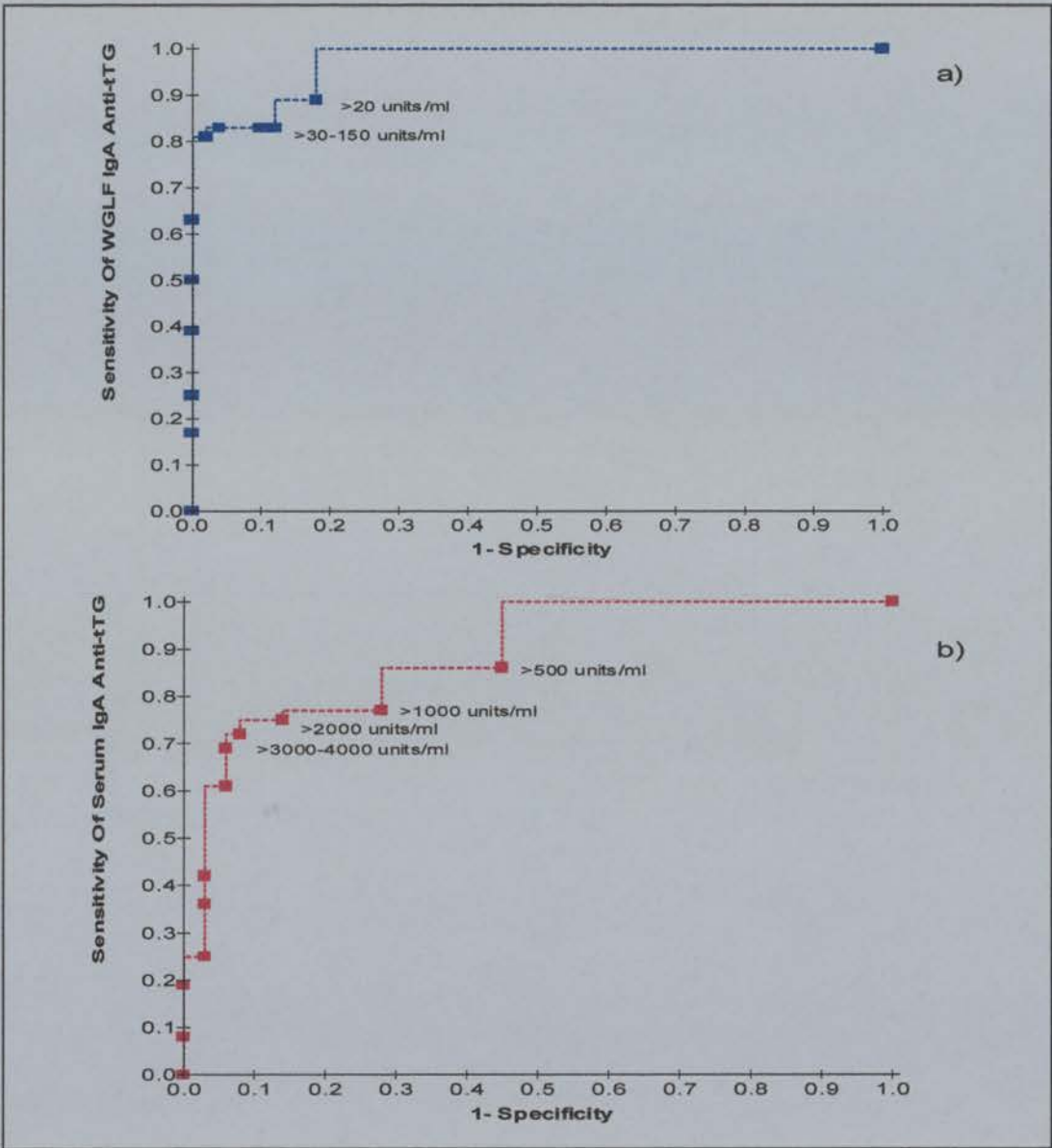


Figure 7.5a & b: Receiver operating characteristic curves of the IgA anti-tTG antibody assay in a) WGLF & b) serum. The assay sensitivity is plotted against the specificity at different cut-off points of IgA anti-tTG antibody concentrations. The optimal cut-off point gives the highest assay sensitivity and specificity. The WGLF assay was more sensitive and specific than the serum assay.

Table 7.3: Coeliac Disease Patients with a Raised IgA Anti-tTG in WGLF but not in Serum

	Age / Gender	WGLF IgA tTG (units/ml)	Serum IgA tTG (units/ml)	WGLF IgA EmA	Serum IgA EmA
1	29 F	125	300	Negative	Equivocal
2	68 F	150	410	Positive	Positive
3	47 M	185	1150	Negative	Negative
4	71 F	1305	2500	Positive	Positive
5	43 F	1680	830	Negative	Negative

WGLF & Serum IgA EmA:

The IgA EmA was positive in 24/36 (67%) and 25/36 (69%) of WGLF and serum samples respectively. There were 5 discordant results for WGLF and serum IgA EmA in these 36 untreated CD patients. In 2 instances, the IgA EmA was positive in WGLF but negative (n=1) or equivocal (n=1) in serum. In 2 cases, the IgA EmA was negative in WGLF but positive (n=2) in serum. The final discordant result was an equivocal WGLF IgA EmA that was positive in serum (**Table 7.4**). The small intestinal histology showed PVA for patients 1 and 5, and STVA for patients 2,3 and 4 in this table. The concordance rate for WGLF and serum IgA EmA was 86%.

Table 7.4: Discrepancies between IgA EmA Results in WGLF & Serum of Coeliac Disease Patients

Patient	Age / Gender	WGLF IgA EmA	Serum IgA EmA	WGLF IgA tTG (units/ml)	Serum IgA tTG (units/ml)
1	37 F	Positive	Negative	285	8620
2	70 F	Positive	Equivocal	490	6880
3	55 M	Negative	Positive	210	5410
4	44 F	Negative	Positive	25	5680
5	64 F	Equivocal	Positive	570	16100

WGLF & Serum IgA AGA:

WGLF and serum IgA AGA concentrations were raised in 28/36 (78%) and 22/36 (61%) of untreated CD patients respectively. The concordance rate for WGLF and serum IgA AGA was 77%.

7.4.6 Comparison of WGLF IgA Anti-tTG & WGLF Total IgA In Coeliac Disease Patients

There was a very weak correlation between WGLF IgA anti-tTG and WGLF total IgA in the untreated CD patients ($r^2 = 0.25$, $p < 0.002$) (**Figure 7.6**). The correlation was reduced by one outlying result for a patient with a WGLF IgA anti-tTG antibody concentration of 4770 units/ml and a total WGLF IgA concentration of 102 g/l. When this result was excluded, the correlation improved ($r^2 = 0.50$, $p < 0.0001$).

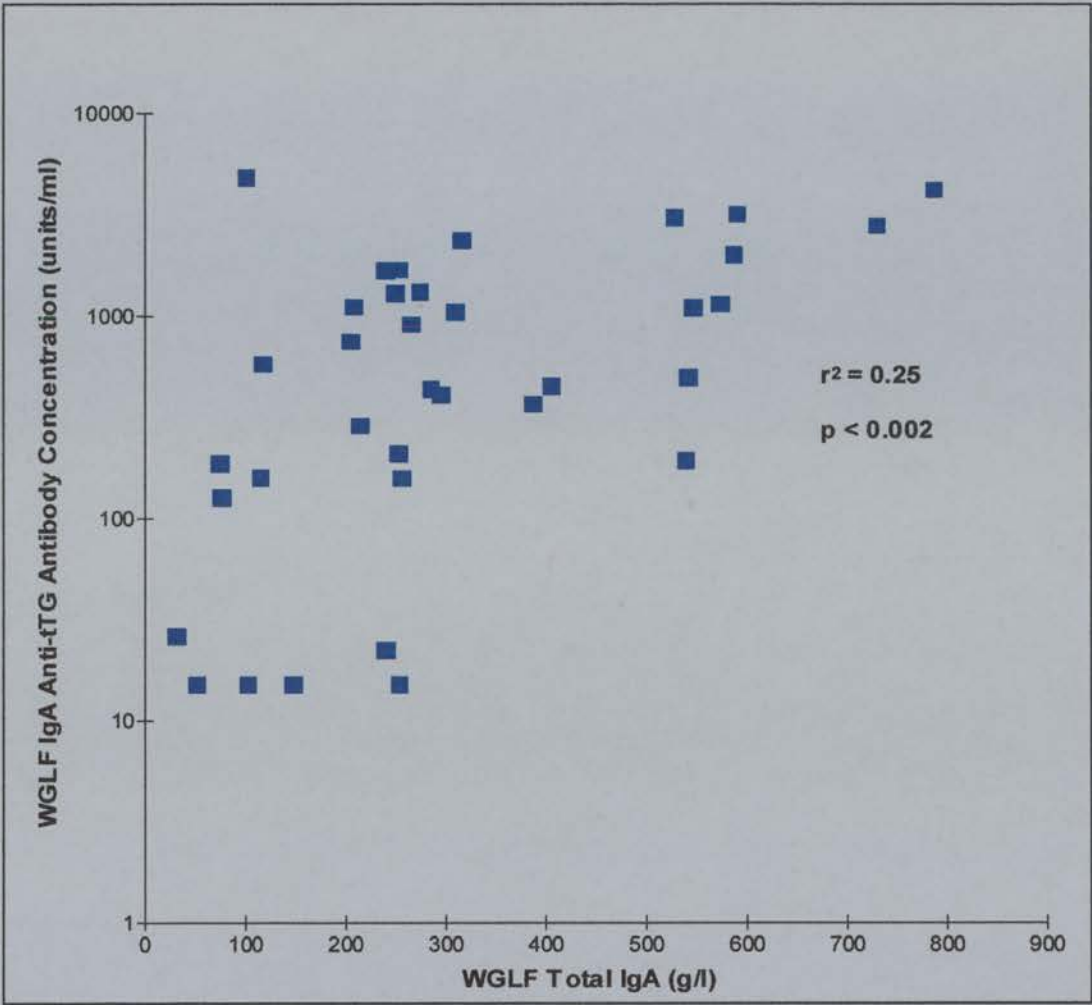


Figure 7.6: Correlation between WGLF IgA anti-tTG antibody & WGLF total IgA

7.4.7 WGLF Total IgG & Albumin

The total IgG was $>10 \mu\text{g/ml}$ in a total of 70 WGLF samples (**Table 7.2**). The number of WGLF samples with a raised total IgG was significantly higher in the IBD subjects than the untreated CD patients ($\chi^2=10.5$, $p<0.001$) or the 49 IBS/healthy volunteer subjects ($\chi^2=29.1$, $p<0.0001$). In the Crohn's disease patients, the WGLF IgA anti-tTG antibody concentration was significantly higher in subjects with a raised WGLF IgG, than in those with a normal WGLF IgG (Mann-Whitney test, $p<0.02$), but this finding was not observed in the UC patients (Mann-Whitney test, $p=0.70$). There was however, no significant difference in WGLF IgA anti-tTG antibody concentrations in the Crohn's disease patients with isolated small ($n=22$) or large bowel ($n=24$) involvement (Mann-Whitney test, $p=0.95$). There was no significant correlation between WGLF anti-tTG antibody concentrations with total IgG ($r^2=0.007$, $p=0.63$) or with WGLF albumin concentrations IgG ($r^2=0.01$, $p=0.49$) in the CD patients. A similar lack of correlation was observed in the IBD patients.

A raised WGLF total IgG and albumin were not typical features of untreated CD patients as this abnormality was only seen in 4 of these patients (**Table 7.5**). A raised ratio of WGL albumin: serum albumin, and WGLF IgG: serum IgG >1 , observed in these 4 patients indicated a protein losing enteropathy. The WGLF IgA anti-tTG antibody concentration was raised in 3 of these patients all of whom had STVA on duodenal biopsies. The other patient had a normal WGLF IgA anti-tTG antibody concentration with only mild PVA on duodenal biopsy. The protein losing enteropathy in this patient, was likely to be due to collagenous colitis which was histologically confirmed (**patient 10 in Table 5.3**).

Table 7.5: Coeliac Disease Patients with a Protein Losing Enteropathy

	WGLF IgG (μg/l)	Serum IgG (g/l)	WGLF: Serum IgG Ratio	WGLF Albumin (μg/l)	Serum Albumin (g/l)	WGLF: Serum Albumin Ratio	WGLF IgA anti-tTG (units/ml)
1	12	10.2	1.17	41	35	1.17	446
2	26	15.1	1.72	86	33	2.61	1137
3	17	5.23	3.25	65	29	2.24	15
4	78	7.06	11.05	150	27	5.56	486

Duodenal mucosal histology showed STVA in patients 1, 2 and 4, and PVA in patient 3. The serum IgA anti-tTG antibody concentrations for patients 1-4 were 8250, 81600, 300 and 6880 units/ml respectively.

7.5 Discussion

This chapter demonstrates that IgA anti-tTG antibodies can be detected in the WGLF of untreated CD patients, and that the concentrations of IgA anti-tTG antibodies and IgA AGA in WGLF were significantly higher in untreated CD patients than in other GI diseases or in healthy volunteers. The measurement of IgA anti-tTG antibodies and IgA AGA in WGLF were more sensitive (83% and 78%) at detecting untreated CD than the measurements in serum (72% and 61%) respectively. In contrast, others have found that serum IgA AGA was superior to mucosal IgA AGA in detecting CD (Kelly et al. 1991).

Relatively equal numbers of untreated CD patients had a positive IgA EmA in WGLF (67%) and in serum (69%), although there were 5 discordant results.

The WGLF IgA anti-tTG antibody concentrations were raised in 5 untreated CD patients with normal serum IgA anti-tTG antibody concentrations, indicating that there was a local gut immune response to tTG that was independent of the systemic response. Others have also found that mucosal IgA antibodies may be found without the corresponding antibodies being detectable in the serum (Engstrom et al. 1992). It would have been useful to repeat the WGL procedure in the CD patients following treatment with a GFD to assess if IgA anti-tTG antibodies persisted in the WGLF. Others have previously found that intestinal, but not serum IgA AGA, persist in treated CD patients even after histological recovery (O'Mahony et al. 1991; Kelly et al. 1991; Lavo et al. 1992). It was disappointing, but understandable, that none the CD patients approached agreed to undergo a second WGL procedure following dietary treatment for research purposes only.

In CD samples, there was a significant correlation between WGLF IgA anti-tTG antibody and WGLF total IgA concentrations. There was however, no correlation between WGLF and serum IgA anti-tTG antibody concentrations, or between WGLF IgA anti-tTG antibody and WGLF IgA AGA concentrations. The lack of a positive correlation between WGLF and serum IgA anti-tTG antibody concentrations was further evidence for the compartmentalisation of the mucosal and systemic immune responses. It also suggested that the presence of IgA anti-tTG antibodies in the WGLF of untreated CD patients was not simply due to secretion from the serum into the gut lumen. Others have found an inverse relationship between mucosal tTG and serum TG activity, such that while there was an increase in mucosal tTG in untreated CD patients, the serum TG activity was reduced (D'Argenio et al. 1989). The absence of a relationship between WGLF IgA anti-tTG antibody and WGLF IgA AGA concentrations, questions the

requirement for a high intra-luminal gliadin concentration for the formation of anti-tTG antibodies.

These findings are supported by reports of a lack of a correlation between jejunal fluid and serum Igs (Lancaster-Smith et al. 1974; Labrooy et al. 1980), but others have found a significant positive correlation (Lavo et al. 1992; O'Mahony et al. 1991). Supporting the theory that serum IgA does not contribute significantly to WGLF IgA, are the findings that WGLF IgA has been found to be predominantly of secretory type in both CD and control groups (O'Mahony et al. 1990), and that about 98% of polymeric IgA is locally produced in the gut wall (Jonard et al. 1984). Secretory IgA has however also been demonstrated in the serum of untreated CD patients, as well as in IBD and healthy controls (Volta et al. 1990; Thompson et al. 1969). This suggests that the two compartments are not mutually exclusive, and that there may be an over-spill from the intestinal mucosal into the circulation (Volta et al. 1990).

The WGLF IgA anti-tTG antibody concentrations were raised in 10/264 (4%) of all control patients, but the WGLF IgA EmA was negative in all of these samples. Six of these 10 subjects had small intestinal biopsies performed and the biopsies were all normal except in 2 IBD patients. One was a UC patient with severe PVA and the other was a Crohn's disease patient with STVA. Neither of these two patients would have been diagnosed as having co-existent CD using any of the serum antibody tests. WGLF IgA anti-tTG antibodies were therefore highly specific for untreated CD with a false positive detection rate of only 8/264 (3%) controls which is low considering that tTG is known to be widely distributed in the gut submucosa (Patel et al. 1985).

The presence of raised WGLF IgA anti-tTG antibody concentrations in some of the control subjects may have also resulted from the interaction between tTG and extra-cellular matrix components (fibronectin, collagen II, V, XI, and procollagen II) with

which tTG is known to interact (Upchurch et al. 1987). As discussed in **section 2.4.4**, mucosal tTG activity is reduced in both acute and chronic colitis and the level of the reduction was related to the severity of the mucosal damage (D'Argenio et al. 1992). This may also explain why WGLF IgA anti-tTG antibody concentrations were only raised in 4 of the IBD patients studied. The higher specificity of the IgA EmA test was also observed in serum, and is likely to be due to the use of a human tissue substrate in comparison with the guinea pig liver source of tTG.

A further interesting observation was the finding that the Crohn's disease patient with isolated active duodenal and jejunal inflammation, had elevated WGLF and serum IgA AGA concentrations. This was likely to be due to an increased small intestinal permeability to dietary gluten. It was interesting that despite active mucosal inflammation, this patient did not have raised IgA anti-tTG antibody concentrations in WGLF or serum. This suggests that the production of IgA anti-tTG antibodies, requires additional factors other than the availability of gluten, and an inflamed mucosa for the mucosal production of IgA anti-tTG antibodies.

An elevated WGLF total IgG >10 µg/ml was predominantly seen in patients with IBD, but was also found in 4 CD patients, one of whom had collagenous colitis. The WGLF IgA anti-tTG antibody concentration was significantly higher in active than inactive Crohn's disease patients, but this was not observed in UC patients. The WGLF IgA anti-tTG antibody concentrations, however were not significantly higher in Crohn's disease patients with proximal, as opposed to distal, disease distributions. This finding was in agreement with an earlier report showing that levels of food antibodies in the serum and WGLF of Crohn's disease patients with proximal and distal distributions were similar, but that the WGLF IgA was higher in patients with active disease (Mwantembe, 1992).

The finding of a low WGLF total IgG in untreated CD patients and healthy volunteers was not surprising as the number of IgG secreting plasma cells are known to be low, both in untreated CD (Colombel et al. 1990; Lancaster-Smith et al. 1974) and in healthy individuals (Crabbe et al. 1965). The concentration of IgG AGA in jejunal secretions has also been shown to be low in untreated CD (Colombel et al. 1990; Lavo et al. 1992). Some authors have found a raised number of IgG producing plasma cells in CD mucosa (Scott et al. 1980; Scott et al. 1984). The absence of a facilitated transport system to the lumen for IgG and the fact IgG is not protected by the secretory component (Walker and Isselbacher, 1977), would make IgG less prominent even if the number of IgG producing cells were increased. IBD patients are known to have a marked increase in the number of IgG-producing cells in the lamina propria which correlate with disease activity (Rosekrans et al. 1980). As albumin is exclusively plasma-derived, and the WGLF albumin correlates positively with WGLF IgG, it suggests that WGLF IgG is predominantly due to leakage across an inflamed mucosa (O'Mahony et al. 1991; O'Mahony et al. 1990; Jonard et al. 1984; Wood et al. 1987). As a protein losing enteropathy was seen in only 4 of the untreated CD patients studied, it also suggests that intra-luminal IgA anti-tTG antibodies are not secreted from the serum, but are locally produced in the gut mucosa.

The likely mechanism for the local production of IgA anti-tTG antibodies was through the specific interaction between gliadin and submucosal tTG, which enhances the presentation of gliadin to the local immune system in the gut as previously discussed in **section 2.4.3**. The significantly higher jejunal mucosal tTG activity in untreated CD than in IBD patients or controls (Bruce et al. 1985), and the reduced mucosal activity in chemical-induced colitis (D'Argenio et al. 1992), may in part explain why anti-tTG antibodies are very specific to CD. Also, as the enzyme has a major role in wound healing (Upchurch et al. 1991; Upchurch et al. 1987) which is also high-lighted by its increased activity in repairing than damaged tissue (D'Argenio et al. 1992), the presence

of antibodies against tTG may in fact be counter-productive to the regeneration processes in the gut mucosa.

The use of WGL for the study of gut mucosal immunity had some limitations. Although the addition of protease inhibitors reduced the level of proteolysis after WGLF collection, proteins leaking proximally into the gut lumen may have been destroyed during their transit (1-2 hours) through the gut (Choudari et al. 1993; Ferguson et al. 1995). The technique was also unable to determine the level of antibody production from the intestine, or to determine if substances were plasma-derived (Ferguson et al. 1995). It is possible that differences in mucosal antibody production in proximal and distal Crohn's disease were not observed because the WGL procedure perfused the whole gut. For the patient, the procedure was time-consuming and the PEG solution was unpalatable. Despite these limitations, this study demonstrated some novel findings in the mucosal responses to tTG and to gliadin in CD patients and those with other GI disorders. In the next chapter, the relationship between serum and WGLF serology will be compared with morphometric features and DQ2 typing.

CHAPTER 8: RELATIONSHIP BETWEEN SERUM & WGLF COELIAC SEROLOGY WITH MORPHOMETRIC FEATURES & HLA-DQ2 TYPING

8.1 Introduction

At the time this study was undertaken, there were no studies comparing the concentration of IgA anti-tTG antibodies with the severity of the histological lesion or morphometric measurements. Recently, Rostami showed that raised IgA anti-tTG antibody concentrations correlate positively with the severity of the histological lesion with sensitivities of 20%, 66% and 100% in subjects with PVA, STVA and TVA respectively (Rostami et al. 1998). He has also reported a similar correlation for IgA EmA and AGA, and suggested that all of these tests may be negative in patients with less severe enteropathy (Rostami et al. 1999a; Rostami et al. 1998; Rostami et al. 1998). It was also reported that the IEL count was significantly higher in patients with positive serology (anti-tTG antibodies, IgA EmA and IgA AGA), than those with negative serology (Rostami et al. 1998). He also showed that the sugar absorption test ratio was raised in 85%, 90% and 100% of patients with PVA, STVA and TVA respectively (Rostami et al. 1998). There are however no reports comparing the sugar test result directly with serological markers to determine if individuals with a more permeable mucosa were more likely to have higher serological results.

The diagnosis of CD is based on the finding of varying degrees of villous atrophy in small intestinal mucosal biopsies. As discussed in **section 2.3.4**, there are five main lesions described (Marsh types 0-4) which represent the full spectrum of gluten-sensitive mucosal lesions (Marsh, 1992). This classification system is not used in

routine reporting by pathologists. In practice, the type 1 lesion corresponds to a normal mucosal architecture with a marked infiltration of IELs. The types 2, 3 and 4 lesions generally correspond respectively with the PVA, STVA and TVA terminology used in routine reporting.

As previously discussed in **section 2.3.4**, there is strong evidence of a genetic susceptibility in CD, particularly with the HLA DQ2 heterodimer, DQ2 ($\alpha 1^*0501$, $\beta 1^*0201$) (Lundin et al. 1993; Sollid et al. 1989), which is present in about 90-95% of British CD patients (Sollid and Thorsby, 1993; Marsh et al. 1993). HLA DQ2-restricted gliadin-specific T helper cells are present in small intestinal biopsies of CD patients (Molberg et al. 1997), and these cells play a key role in the production of anti-tTG antibodies as previously described in **section 1.5.3**. Of importance, Molberg *et al.* have shown that the enhanced DQ2 recognition of the tTG-gliadin complexes occurs only by gut-derived, but not peripheral, gliadin-specific T cells (Molberg et al. 1998). The local production of anti-tTG antibodies in the gut submucosa may lead to their secretion into the gut lumen.

The aims of this chapter therefore were to investigate the association between serology with HLA-DQ2 status, histology and intestinal permeability. This was in order to determine if the concentrations of serological markers reflected the severity of the intestinal lesion. The serum and WGLF antibody concentrations of IgA anti-tTG, IgA EmA and IgA AGA, were compared with HLA-DQ2 status, morphometry and L/R ratio in untreated CD patients. The histological parameters studied included the Marsh grade, IEL counts, CD3 and δ/λ T cell counts, as well as villous height and crypt depth. The corresponding Marsh grade and HLA-DQ2 status were also compared to determine if this haplotype was associated with more severe histological lesions.

8.2 Patients

The same 53 prospectively diagnosed CD and DH patients (39 females: 14 males; age range 22-77 years - median 51 years) presenting between October 1997 and April 1999 discussed in **section 6.2** were investigated in this arm of the study. Serological assays were performed in all 53 patients but a WGLF sample could be obtained from only 36 of these patients.

8.3 Methods & Statistical Analysis

The serum and WGLF IgA anti-tTG antibodies, IgA EmA and IgA AGA were measured in all samples using methods previously described in **sections 3.3, 4.4.3, and 4.2.3**. The HLA-DQ2 typing was performed on 43 of the 53 CD patients studied using the technique described in **sections 4.7 and 6.3**. All sugar permeability tests were performed as part of the routine GI laboratory service using the method described in **section 4.8**. The normal L/R reference range was < 0.04 .

Endoscopic duodenal biopsies were obtained from all CD patients using 37K rat-tooth, biopsy forceps (SB-37K-1, Olympus Endotherapy). All H&E stained biopsy sections were examined with assistance from a senior pathologist, Dr. Margaret McIntyre, and were graded (types 0-4) using the Marsh criteria (Marsh, 1992). Histological methods for the quantification of IELs, CD3 and γ/δ cells were previously described in **sections 4.4.6 and 4.4.7** respectively. The micro-dissection technique used to measure the villous length, crypt depth and number of mitotic figures was described in **section 4.5.1** and the normal reference ranges were 500-1100 μm , 150-300 μm and 1-12 respectively.

The statistical methods applied were described in **section 4.9**. The χ^2 test was used to compare the number of DQ2 positive CD patients with different severity of histological lesions as graded by the Marsh criteria. This test was also used to compare the serum

and WGLF IgA anti-tTG antibody, IgA EmA and IgA AGA results with Marsh grading, and the number of CD patients with positive serum and WGLF IgA EmA results in DQ2 positive and negative CD patients. The Mann-Whitney test was used to compare serum and WGLF IgA anti-tTG antibody, IgA AGA and IgA EmA in DQ2 positive and negative individuals. Pearson's correlation was used to compare serum and WGLF IgA anti-tTG antibody and IgA AGA concentrations with corresponding IEL, CD3 and γ/δ counts, as well as measurements of crypt depth, villous height and crypt mitoses. This test was also used to compare serum and WGLF antibody measurements with the matched L/R results.

8.4 Results

8.4.1 Comparison between HLA-DQ2 Typing & Marsh Grade

The HLA-DQ2 typing was determined in 36/43 CD patients as previously described in **section 6.4.2**. The DQ2 was positive in 22 and negative in 14 of these samples. The frequency of DQ2 positive patients with Marsh grades 1 and 2 lesions, were compared with the frequency of DQ2 positive patients with grades 3 and 4 lesions. This showed that there was no significant association between the DQ2 status and histological severity ($\chi^2=0.058$, $p=0.81$) (**Table 8.1**).

Table 8.1: HLA-DQ2 Type Compared with Histological Severity

Marsh Grade	DQ2 Positive	DQ2 Negative
1	1	2
2	6	3
Grades 1 & 2	7	5
3	15	8
4	0	1
Grades 3 & 4	15	9
Total	22	14

8.4.2 Comparison between HLA-DQ2 Typing with Antibodies in Serum & WGLF

The DQ2 status was known in 36 CD patients in whom the serum IgA anti-tTG antibody, IgA EmA and IgA AGA concentrations were measured. Of these 36 subjects, 22 were DQ2 positive. The DQ2 status was known in 27 CD patients in whom the corresponding antibodies in WGLF were measured. Of these individuals, 15 were DQ2 positive. The DQ2 status was compared with the antibody concentrations in serum and WGLF.

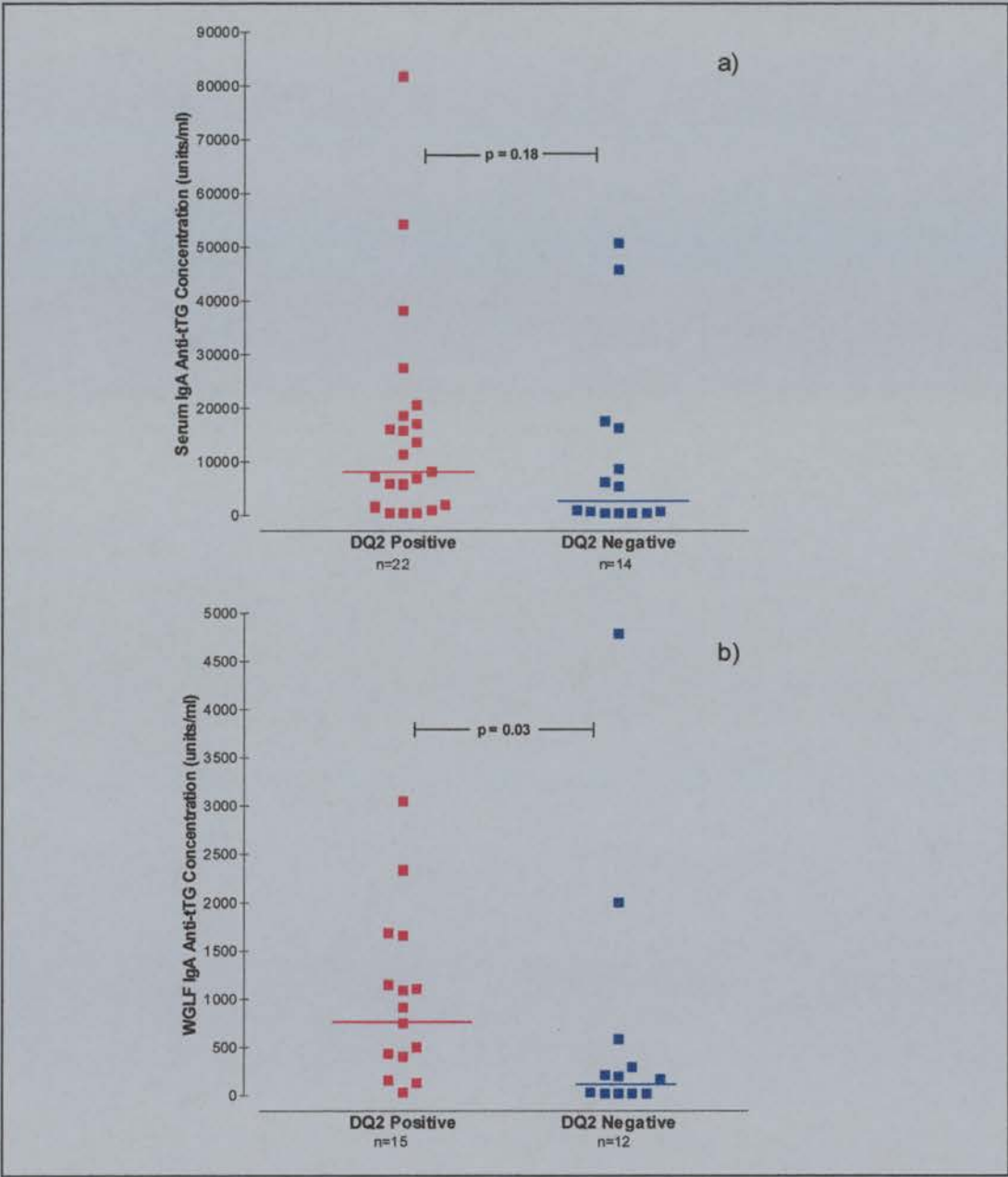
Serum & WGLF IgA anti-tTG antibodies:

The serum and WGLF IgA anti-tTG antibody concentrations in HLA-DQ2 positive and negative patients are shown in **Table 8.2**. The IgA anti-tTG antibody concentrations were significantly higher in the WGLF, but not in the serum, of DQ2 positive than DQ2 negative CD patients (Mann-Whitney test: serum $p=0.18$, WGLF $p=0.03$) (**Figures 8.1a & 8.1b**). There was no significant difference in the frequency of DQ2 positive patients with a raised serum or WGLF IgA anti-tTG antibody concentration (serum: $\chi^2=1.92$, $p=0.17$, WGLF: χ^2 with Yates correction = 2.92, $0.05 < p < 0.1$).

Two DQ2 negative untreated CD patients had very high WGLF IgA anti-tTG antibody concentrations of 1995 units/ml and 4770 units/ml. The first subject was a 61 year old lady with a learning disability who had presented with a one year history of weight loss of 3 stones, anorexia and fatigue, but no alteration in bowel habit. All of the serum antibodies were positive: the IgA anti-tTG antibody concentration was raised at 17400 units/ml, IgA AGA was 3025 units/ml, and the IgA EmA was positive. The L/R ratio was raised at 0.104 and the duodenal biopsy histology showed PVA. The other patient was a 52 year old man who presented with a 1 year history of weight loss, fatigue and abdominal bloating. The IgA anti-tTG antibody concentration was raised at 17400 units/ml and the IgA EmA was positive, but the IgA AGA level was just below the upper reference limit at 29 units/ml. The L/R ratio was raised at 0.072 and small intestinal biopsies showed STVA. The clinical presentation, serology, L/R ratios and histology all supported the diagnosis of CD in these 2 DQ2 negative individuals.

Table 8.2: HLA-DQ2 Status Compared with Serum & WGLF IgA Anti-tTG Antibody Concentrations

	DQ2 Positive	DQ2 Negative
Number of Patients with Serum IgA Anti-tTG >2950 units/ml	16/22 (73%)	7/14 (50%)
Median Serum IgA Anti-tTG concentration (units/ml)	9630	3175
Number of Patients with WGLF IgA Anti-tTG >110 units/ml	14/15 (93%)	7/12 (58%)
Median WGLF IgA Anti-tTG concentration (units/ml)	905	175
Total Number of Patients	36	27



Figures 8.1a & b: Comparison between a) serum & b) WGLF IgA anti-tTG antibody concentrations in HLA DQ2 positive & negative CD patients. The IgA anti-tTG antibody concentrations were significantly higher in the WGLF but not the serum of DQ2 positive compared to DQ2 negative patients.

Serum & WGLF IgA EmA antibodies:

The number of CD patients with positive serum and WGLF IgA EmA results were compared in DQ2 positive and negative CD patients (**Table 8.3**). Using the χ^2 test with Yates correction, this showed that DQ2 positive patients did not have a significantly higher frequency of positive IgA EmA results in serum or in WGLF (serum: $\chi^2=2.72$, $0.5>p>0.1$, WGLF: $\chi^2=3.06$, $0.1>p>0.05$).

Serum & WGLF IgA AGA antibodies:

There was no significant difference in IgA AGA concentrations in serum or WGLF of DQ2 positive and negative CD patients (Mann-Whitney test: serum $p=0.32$, WGLF $p=0.94$).

Table 8.3: Frequency of Serum & WGLF IgA EmA Results in DQ2 Positive & Negative CD Patients

	Number of Patients with a Positive Serum IgA EmA	Number of Patients with a Positive WGLF IgA EmA
DQ2 Positive Patients	18/22 (82%)	12/15 (80%)
DQ2 Negative Patients	7/14 (50%)	5/12 (40%)
Total Number of Patients	25/36	17/27

8.4.3 Comparison between Marsh Grade with Serum & WGLF Antibodies

The Marsh grade was determined for all 53 prospectively diagnosed CD patients. The serum IgA anti-tTG antibody, IgA EmA and IgA AGA concentrations were measured in all of these patients. The corresponding antibodies were measured in the WGLF from the 36 patients who completed the WGL procedure. The severity of the histological lesion was compared with serum and WGLF antibody levels to determine if high antibody levels were associated with more severe pathology.

Serum & WGLF IgA anti-tTG antibodies:

The number of patients with raised serum and WGLF IgA anti-tTG antibody concentrations for each Marsh grade is shown in **Table 8.4**. The number of patients with a raised serum and WGLF IgA anti-tTG antibody concentration in Marsh grades 1 and 2 were compared with the number of patients with Marsh grades 3 and 4 lesions. This showed that patients with Marsh grades 3 and 4 lesions did not have significantly higher serum or WGLF IgA anti-tTG antibody concentrations than patients with Marsh grades 1 and 2 lesions (serum: $\chi^2 = 0.097$, $p > 0.5$; χ^2 test with Yates correction: WGLF $\chi^2 = 0.84$, $p > 0.5$). A χ^2 test for trend (at 3 degrees of freedom) showed no significant increase in the frequency of raised IgA anti-tTG antibody concentrations from Marsh grades 1 to 4 ($\chi^2 = 2.19$, $0.5 > p > 0.1$). The distribution and median concentrations of serum and WGLF IgA anti-tTG antibodies in the Marsh grades are shown in **Figures 8.2a & b**.

Table 8.4: Frequency of Raised Serum & WGLF IgA Anti-tTG Antibody Concentrations Compared with Marsh Grade

Marsh Grade	Number of Patients with a Serum IgA Anti-tTG >2950 units/ml	Number of Patients with a WGLF IgA Anti-tTG >110 units/ml
1	2/5 (40%)	1/3 (33%)
2	12/17 (71%)	12/13 (92%)
3	20/30 (67%)	16/19 (84%)
4	1/1 (100%)	1/1 (100%)
Total Number of Patients	35/53	30/36

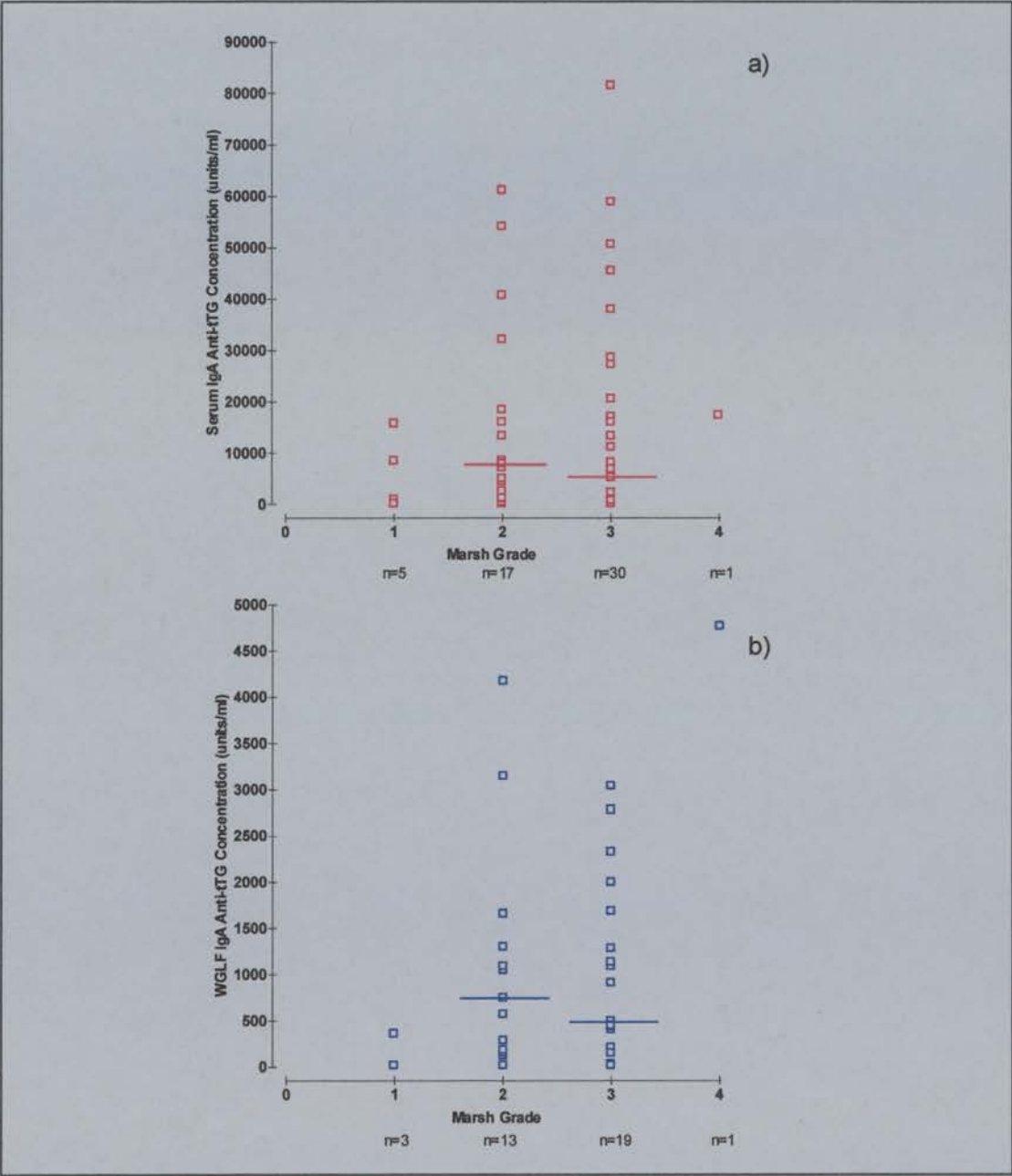


Figure 8.2a & b: Distribution of serum & WGLF IgA anti-tTG antibody concentrations in the Marsh grades.

Serum & WGLF IgA EmA:

The frequency of patients with positive serum and WGLF IgA EmA results in each Marsh grade is shown in **Table 8.5**. The number of patients with a positive IgA EmA, in serum and WGLF, in patients with Marsh grades 1 and 2 lesions, were compared with those with Marsh grades 3 and 4 lesions. This showed that patients with more severe histological lesions (Marsh grades 3 and 4) did not have a significantly higher frequency of positive IgA EmA results in serum ($\chi^2=1.08$, $p=0.3$) or in WGLF ($\chi^2=0.66$, $p=0.42$).

Table 8.5: Serum & WGLF IgA EmA Compared with Marsh Grade

Marsh Grade	Number of Patients with a Positive Serum IgA EmA	Number of Patients with a Positive WGLF IgA EmA
1	2/5 (40%)	1/3 (33%)
2	13/17 (76%)	9/13 (69%)
3	24/30 (80%)	14/19 (74%)
4	1/1 (100%)	1/1 (100%)
Total Number of Patients	40/53	25/36

Serum & WGLF IgA AGA:

The frequency of patients with a raised serum and WGLF IgA AGA concentration for each Marsh grade is shown in **Table 8.6**. The number of patients with a raised serum and WGLF IgA AGA concentration in Marsh grades 1 and 2 were compared with the number of patients with Marsh 3 and 4 lesions. This showed that there was no significant increase in the number of patients with a raised IgA AGA in serum or WGLF in patients with more severe histological lesions (Marsh grade 3 and 4) (serum: $\chi^2=0.42$, $p>0.5$; χ^2 test with Yates correction, WGLF: $\chi^2=0.28$, $p>0.5$).

**Table 8.6: Frequency of Raised Serum & WGLF IgA AGA Concentrations
 Compared with Marsh Grade**

Marsh Grade	Number of Patients with a Serum IgA AGA >30 units/ml	Number of Patients with a WGLF IgA AGA >5 units/ml
1	3/5 (60%)	2/3 (67%)
2	10/17 (59%)	10/13 (78%)
3	21/30 (70%)	16/19 (84%)
4	0/1 (0%)	1/1 (100%)
Total Number of Patients	34/53	29/36

8.4.4 Comparison between IEL Counts with Serum & WGLF Antibodies

IEL counts were measured in 51 of the 53 prospectively diagnosed CD patients. The IEL count could not be measured in 2 patients whose duodenal biopsies did not have sufficient intact epithelium. Serum antibody results were available for all of these 51 patients. Of the 36 CD patients who completed a WGL, the IEL count could be quantified in 35 samples.

Serum & WGLF IgA anti-tTG antibodies:

Comparison between the IEL counts with the corresponding IgA anti-tTG antibody concentrations in serum and WGLF showed that there was no significant correlation between IEL counts and serum or WGLF IgA anti-tTG antibody concentrations (serum: $r^2=0.02$, $p=0.26$, WGLF: $r^2=0.04$, $p=0.28$) (**Figures 8.3a & 8.3b**).

Serum & WGLF IgA EmA:

The IEL counts were compared with the corresponding IgA EmA results in serum and WGLF. This showed that there was no significant increase in the frequency of positive serum or WGLF IgA EmA results in patients with higher IEL counts (**Table 8.7**). There was no significant increase in the number of patients with a positive serum IgA EmA in patients with IEL counts $>$ and $< 80/\text{mm}$ (χ^2 test with Yates correction; serum: $\chi^2=1.81$, $0.5>p>0.1$; WGLF: $\chi^2=1.42$, $0.5>p>0.1$).

Serum & WGLF IgA AGA:

The IEL counts were compared with the corresponding IgA AGA concentrations in serum and WGLF. This showed that there was no significant correlation between IEL

counts and IgA AGA concentrations in serum or WGLF (serum: $r^2=0.003$, $p=0.68$, WGLF: $r^2=0.04$, $p=0.26$).

Table 8.7: Serum & WGLF IgA EmA Compared with IEL Count

IEL Count/mm	No. of Patients with a Positive Serum IgA EmA	No. of Patients with a Positive WGLF IgA EmA
<40	2/4 (50%)	1/2 (50%)
41-80	21/28 (75%)	13/20 (65%)
81-120	10/13 (77%)	7/10 (70%)
>121	6/6 (100%)	3/3 (100%)
Total Number of Patients	39/51	24/35

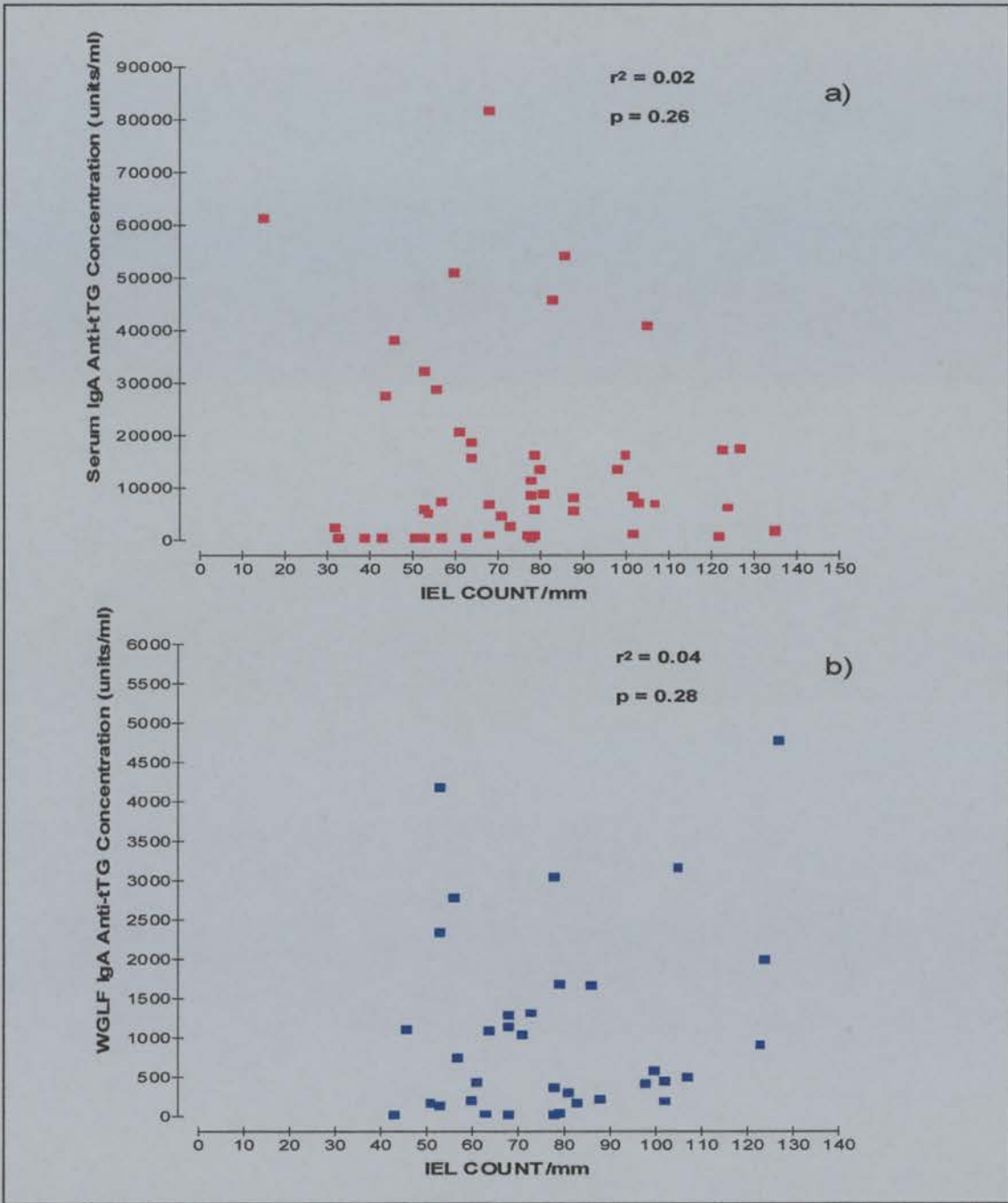


Figure 8.3a & b: Correlation between IEL Count & Serum & WGLF IgA anti-tTG antibody concentrations

8.4.5 Comparison Between CD3 & γ/δ T Cell Counts With Serum & WGLF IgA Anti-tTG Antibody Concentrations

The IgA anti-tTG antibody concentrations in serum (n=19) and WGLF (n=16) were compared with the corresponding epithelial CD3 cell counts. This showed that there was no correlation between the CD3 count and the anti-tTG antibody concentrations either in serum ($r^2=0.007$, $p=0.73$) or in WGLF ($r^2=0.048$, $p=0.41$). There was also no correlation between γ/δ T cell counts and the antibody concentration in serum ($r^2=0.14$, $p=0.12$) or in WGLF ($r^2=0.0007$, $p=0.97$).

8.4.6 Comparison between Morphometric Measurements with Serum & WGLF IgA Anti-tTG Antibody Concentrations

Crypt depth compared with serum & WGLF IgA anti-tTG antibodies:

Measurements of the crypt depth could be made in 26 of the duodenal biopsy specimens. The serum and WGLF IgA anti-tTG antibody concentrations were available for 26 and 20 subjects respectively. Comparison between the crypt depth measurement with the corresponding IgA anti-tTG antibody concentrations in serum, but not WGLF, showed a significant but weak positive correlation (serum: $r^2=0.2$, $p<0.02$, WGLF: $r^2=0.06$, $p=0.27$) (Figure 8.4a & 8.4b).

Villous length compared with serum & WGLF IgA anti-tTG antibodies:

Measurements of the villous length could be made in 20 of the duodenal biopsy specimens. The serum and WGLF IgA anti-tTG antibody concentrations were available for 20 and 16 subjects respectively. Comparison between the villous length measurement with the corresponding IgA anti-tTG antibody concentrations in serum and WGLF showed no significant correlation (serum: $r^2=0.16$, $p=0.08$, WGLF: $r^2=0.05$, $p=0.41$).

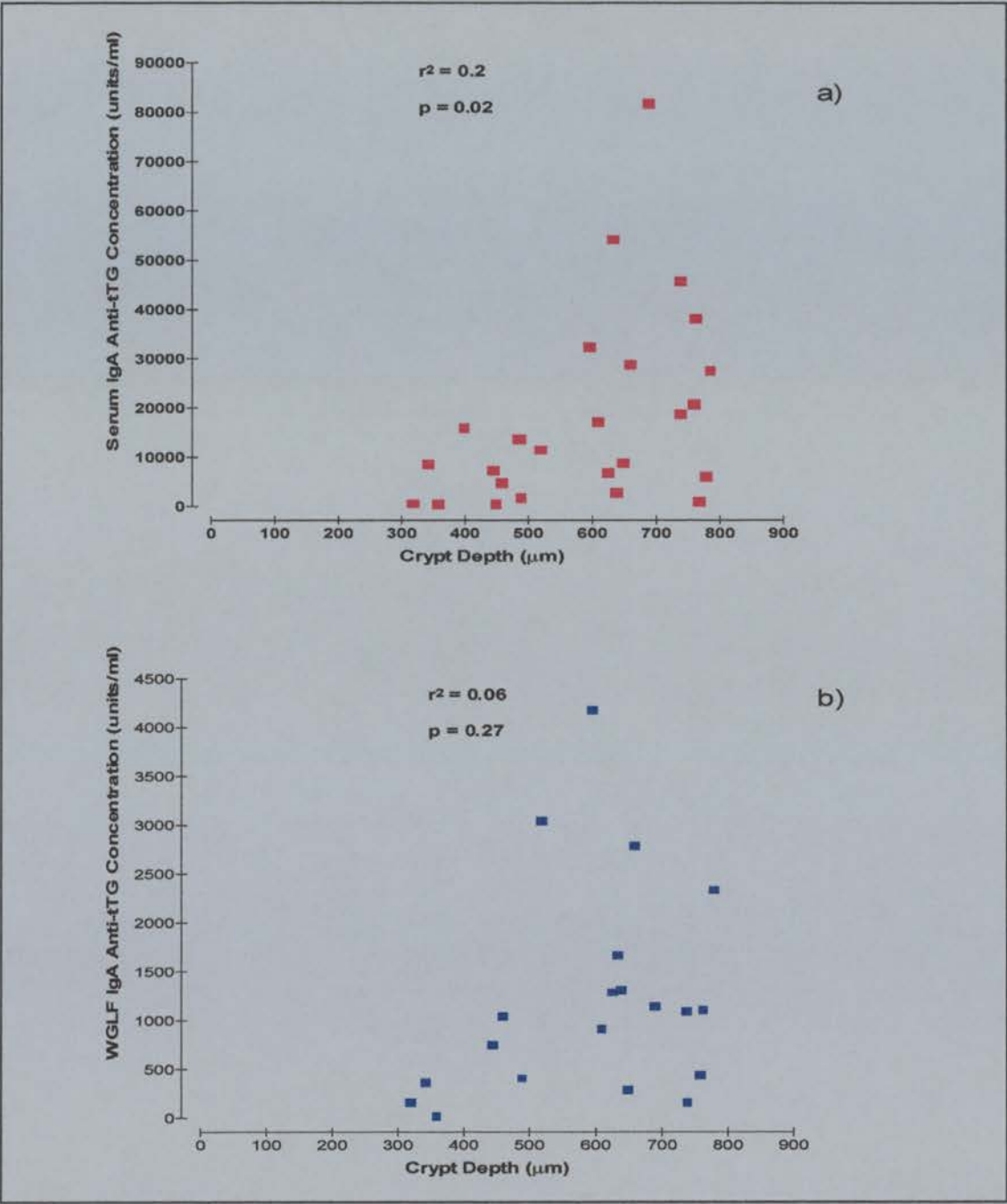


Figure 8.4a & b: Correlation between crypt depth with serum & WGLF IgA anti-tTG antibody concentrations

Number of crypt mitoses compared with serum & WGLF IgA anti-tTG antibodies:

The number of crypt mitoses could be quantified in 24 duodenal biopsies. The serum and WGLF IgA anti-tTG antibody concentrations were available for 24 and 19 of these subjects respectively. Comparison between the number of crypt mitoses and antibody concentrations in serum and WGLF showed no significant correlation (serum: $r^2=0.0001$, $p=0.96$, WGLF: $r^2=0.004$, $p=0.79$).

8.4.7 Comparison between L/R Ratio with Serum & WGLF Antibodies

A sugar permeability test was performed in 48/53 of the prospectively diagnosed CD patients. Of the 36 CD patients completing the WGLF procedure, a sugar permeability test was performed in 35 patients. The L/R ratios were compared in subjects with and without raised concentrations of IgA anti-tTG antibodies, EmA and AGA in serum and WGLF.

Serum & WGLF IgA anti-tTG antibodies:

Comparison between serum and WGLF IgA anti-tTG antibody concentration with L/R showed no significant positive correlation (serum: $r^2=0.0009$, $p=0.84$; WGLF: $r^2=0.001$, $p=0.85$).

Serum & WGLF IgA EmA:

The numbers of patients with a positive serum or WGLF IgA EmA at different intervals of L/R ratios are shown in **Table 8.8**. Using an arbitrary L/R ratio cut-off point of 0.08, there were equal numbers of subjects with a positive serum IgA EmA with ratios above and below 0.08. For the same L/R intervals, there was no significant difference in the

number of patients having a positive WGLF IgA EmA (χ^2 test with Yates correction: $\chi^2=0.133$, $p>0.5$).

Serum & WGLF IgA AGA:

Comparison between serum and WGLF IgA anti-tTG antibody concentration with L/R showed no significant positive correlation (serum: $r^2=0.003$, $p=0.72$; WGLF: $r^2=0.03$, $p=0.33$).

Table 8.8: Serum & WGLF IgA EmA Compared with L/R Ratio

L/R Interval	No. of Patients with a Positive Serum IgA EmA	No. of Patients with a Positive WGLF IgA EmA
<0.04	3/5 (60%)	2/5 (40%)
0.041-0.06	6/8 (75%)	5/7 (71%)
0.061-0.08	9/11 (82%)	6/7 (86%)
0.081-0.1	5/7 (71%)	2/5 (40%)
0.1-0.2	11/12 (92%)	7/8 (88%)
>0.2	2/5 (40%)	2/3 (67%)
Total No. Of Samples	36/48	24/35

8.5 Discussion

The role of antibodies in the pathogenesis of CD is poorly understood. In chapter 6, it was demonstrated that there was no significant difference in the clinical presentation of EmA negative CD patients. The frequency of a positive serum IgA EmA result however, was not significantly higher in DQ2 positive than in DQ2 negative patients. The work presented in this chapter was undertaken to investigate any relationship between serum and WGLF antibodies with the severity of the histological lesion.

The results showed that WGLF, but not serum, IgA anti-tTG antibody concentrations were significantly higher in DQ2 positive than DQ2 negative CD patients. This finding suggested that the DQ2 heterodimer was important in the local production of IgA anti-tTG antibodies in the gut. These findings support the recently proposed theory that the recognition of the tTG-gliadin complex by gut-derived, but not peripheral, gliadin-specific T cells, is favoured by DQ2 presentation (Molberg et al. 1998). Interestingly however, the DQ2 positive CD patients in this study did not have more severe small intestinal mucosal lesions. This may indicate that the presence of antibodies does not contribute directly to the mucosal abnormalities characteristic of CD.

In addition, there was no association between serum or WGLF serology with the Marsh grade, IEL, CD3 or γ/δ TCR counts, micro-dissection parameters or L/R ratio, except for the finding of a weak positive correlation between serum IgA anti-tTG antibody concentration and crypt depth. This observation suggests a role for IgA anti-tTG antibodies in the process of crypt hyperplasia. There was however no association between serum or WGLF antibody concentrations and the severity of villous atrophy indicated by measurement of the villous length. This is in keeping with an earlier report that found no significant relationship between mucosal tTG enzyme activities with morphometric measurements in untreated CD patients (Bruce et al. 1985). These

findings are also supported by a recent report that found no association between IgA EmA and histological severity (Dickey et al. 2000). In this prospective study, serum IgA EmA was found to have an overall sensitivity of 78% (69/89). There was a similar sensitivity for PVA (79%) and for STVA/TVA (77%). Others have also reported no association between clinical observations and gut histopathology in CD (Keshavarzian et al. 1987). In this study, of 53 patients undergoing jejunal biopsy on clinical grounds, villous atrophy was identified in only 15 patients. The biopsies in the other 38 subjects were normal.

In contrast, others have found a raised serum IgA anti-tTG antibody (Rostami et al. 1998) and IgA AGA (Rostami et al. 1999b) concentration, as well as a positive serum IgA EmA (Rostami et al. 1999b; Rostami et al. 1999a; Kumar et al. 1989) to be associated with more severe histological lesions. The IgA anti-tTG and EmA were both positive in 66% of patients with STVA, but in only 50% and 20% respectively of patients with PVA (Rostami et al. 1998). It is possible that Rostami found a low IgA EmA sensitivity and a positive correlation between IgA EmA and histological severity because the serum samples were diluted at 1:10 and 1:50 in the EmA assay. In starting at a higher sample dilution, it is possible that CD patients with lower antibody titres have been excluded. Like others (Hallstrom, 1989; Kolho and Savilahti, 1997; Cataldo et al. 1995), a serum dilution of 1:5 was used in this thesis. It is also possible that a significant result may have been found in this study if there were more patients with Marsh grades 1 and 4 lesions.

The presence of Igs in gut secretions from patients with CD is in part thought to be due to an increased intestinal permeability allowing the passage of luminal antigens into the submucosa and triggering an immune response. In this study however, there was no association serum or WGLF antibody concentrations with the intestinal permeability.

There has been much controversy about the role of antibodies in the pathogenesis of CD. IgA cannot initiate inflammatory reactions and is primarily thought to serve a housekeeping role by removing relatively harmless antigens from the circulation that have leaked in from the GI or respiratory tracts (Stokes et al. 1980). The presence of intra-luminal antibodies to gliadin in DH patients with normal small intestinal histology also suggests antibodies are not contributing to the mucosal damage in CD (Marsh, 1992). In addition, CD is common in selective IgA deficiency (Collin et al. 1992; Cataldo et al. 1999), and may occur in severe hypogammaglobulinaemia (Webster et al. 1981), which makes it unlikely that antibodies directly contribute to the mucosal lesion.

A recent report however, has shown for the first time that CD-specific IgA tissue antibodies may have a pathogenic role in producing the characteristic lesion of crypt hyperplasia and villous atrophy (Halttunen and Maki, 1999). Using an *in vitro* cell culture technique, they showed that serum IgA anti-tTG antibodies from untreated CD patients interfered with cellular differentiation (increased epithelial cell proliferation) and contributed directly to mucosal flattening. The mechanism was by disturbing the interaction between fibroblasts and epithelial cells via inhibition of transforming growth factor β (TGF- β), which is required for epithelial differentiation (Nunes et al. 1997). This would result in a lack of differentiation of villous epithelium, which has previously been described in CD (van de Wal et al. 2000). It has also been suggested that in CD patients with SigAD, the IgG class tissue autoantibodies might play a similar role. These findings may explain our observation of a positive association between serum IgA anti-tTG antibody concentration and crypt depth. As previously discussed in **section 2.4.3**, tTG is required for the activation of latent TGF- β as it cross-links the latent TGF- β complex via TGF- β -binding protein 1 to the extracellular matrix (Nunes et al. 1997). A possible role of anti-tTG antibodies in the pathogenesis of CD may therefore be via blocking the activation of TGF- β resulting in the lack of differentiation of villous epithelium observed in CD (Schuppan et al. 1998).

In addition, as tTG has many physiological functions, particularly in cellular growth and differentiation, as well as the processes of tissue repair and remodelling (Upchurch et al. 1987; Nunes et al. 1997; Mirza et al. 1997), antibodies directed against it may prevent the enzyme from performing its normal functions, for example in the restitution of mucosal integrity after injury. It has also been suggested that in the absence of active tTG, the accumulation of cleavage products in apoptotic cells might contribute to the development of autoimmunity (Piacentini and Colizzi, 1999). It is therefore feasible that anti-tTG antibodies are counter-productive and may contribute to the coeliac lesion by impairing mucosal differentiation and healing after injury.

The identification of tTG as the specific tissue autoantigen in CD has significantly advanced the knowledge of the pathogenic mechanisms in CD. Although the findings in this chapter did not show a significant relationship between serological markers in serum or in WGLF with histology or intestinal permeability, there were associations between WGLF and serum IgA anti-tTG antibody concentrations with DQ2 status and crypt depth respectively. These findings were consistent with other reports. Future work in this area should include a comparison of mucosal tTG expression (by immunohistochemistry using specific monoclonal antibodies), IgA anti-tTG antibody concentrations and cytokine levels with histology and morphometry.

CHAPTER 9: GENERAL DISCUSSION

9.1 The Diagnosis of Coeliac Disease

Serological testing has been the focus of much research with the aim of facilitating an inexpensive, safe and non-invasive diagnosis of CD. In my opinion, the role of serological testing is primarily for screening, as in the presence of clinical symptoms, small intestinal biopsy should be the first diagnostic procedure. An initial positive IgA anti-tTG or EmA in these patients however, is useful in monitoring gluten withdrawal, such that if these tests become negative in line with a clinical response, a repeat biopsy is not necessary. Serological screening for CD should be directed towards patients with more subtle symptoms and high risk groups. Although very sensitive, intestinal permeability tests are not used widely for screening as they lack specificity (Maki and Collin, 1997b). ELISA methods are thought to be more practical and less expensive for large-scale screening than immunofluorescent assays (Maki and Collin, 1997b).

As CD is a very heterogeneous disease with regard to its clinical manifestations, histological changes and response to dietary treatment, it is perhaps optimistic to expect that serological methods could identify all gluten-sensitive individuals. It also assumes the presence of a limited number of tissue autoantigens. Our finding of sero-negative patients with histological abnormalities who respond clinically and histologically to gluten withdrawal demonstrates that tTG may not be the only tissue autoantigen in CD. Like others, I found that the PPV of IgA anti-tTG antibodies and IgA EmA were high (98% and 100% respectively). The value of serological testing has been questioned if biopsy is still required to confirm the disease in the presence of positive tissue antibody results (Valdimarsson et al. 1996; Corrao et al. 1994). Others feel that intestinal biopsy is still required to confirm the diagnosis despite the finding of positive serology

(Lerner, 1991; Bode and Gudmand-Hoyer, 1994; Sategna-Guidetti et al. 1997; Sulkanen et al. 1998a; Cataldo et al. 1995; Unsworth, 1996). It is known that the sensitivity of serological tests is reduced by SIgAD and immunosuppressive therapy (Sategna-Guidetti et al. 1997; Unsworth, 1996) and may be negative in milder forms of mucosal damage (Rostami et al. 1999a; Rostami et al. 1998; Dick et al. 1969).

In favour of an initial intestinal biopsy is that it does provide a useful baseline in patients who have an atypical clinical course on a GFD, and some physicians and patients also want repeat biopsies to document recovery (Green and Byfield, 1998). Occasionally however, small intestinal biopsies may be misleading as not all flat lesions represent CD (Shidrawi et al. 1994), and CD may follow a normal biopsy (Maki et al. 1990). The revelation of the wide histological spectrum of CD however, has meant that the early clinical definitions of CD are now outdated. The diagnosis of CD can no longer be restricted to the 30% of subjects with flat lesions and symptomatic malabsorption, but should also include the greater proportion of gluten-sensitive individuals who have subtle intestinal lesions (Marsh, 1992). A new definition of gluten sensitivity has been suggested that is free from the constraints of morphology, for example using a molecular probe based on MHC or TCR structure (Marsh, 1992). In my opinion, intestinal biopsy is still necessary in patients with positive serology as some of these individuals may have normal histology (latent CD). Until one can be confident that a positive serological test indicates CD, then one cannot be reassured by anything less than histology.

In the meantime if we are to rely on performing small intestinal biopsies to diagnose CD, then the technique for obtaining biopsies should be standardised. The small intestinal biopsy audit showed that although jejunal biopsies were larger than duodenal biopsies, when both excellent and good biopsies were combined, both jejunal and duodenal biopsies were adequate for the diagnosis of CD. Although our current practice

was sufficient to permit a diagnosis to be made in the majority of patients, the audit highlighted some deficiencies. In particular, there was a wide variation in the number of endoscopic biopsies being taken and the biopsy forceps used. As there are unavoidable problems in orientating duodenal biopsies before fixation due to their small size, we have recommended that a minimum of 4 duodenal biopsies be taken.

9.2 The Performance of the IgA Anti-tTG ELISA

At the beginning of this project, in-house assays for measuring IgA AGA, IgG AGA, and IgA EmA were already established. The IgA and IgG anti-tTG antibody ELISAs had not yet been developed. The IgA anti-tTG antibody ELISA was developed to provide an objective, semi-quantitative technique of measuring the tissue autoantibody response in CD. This new assay was hoped to be sufficiently sensitive and specific to replace the IgA EmA test. Consideration was given to all stages of development and the performance of the assay was monitored using control samples. Specific methodological issues raised during the course of this work were the exclusion of calcium in the coating buffer and the use of BSA in the blocking solution. The in-house IgA anti-tTG assay was found to be less sensitive but more specific than the commercial kit (65% and 96% versus 92% and 81% respectively). Despite this, the sensitivity and specificity of the in-house assay compared well with other reports. There is still no consensus about the requirement for calcium in the assay, but several authors have recommended that BSA should be excluded as some CD patients may have antibodies that may interfere with the assay (Lock et al. 1999b; Sulkanen et al. 1998b; Troncone et al. 1999).

The significant disadvantages of ELISA methods however are that they are difficult to standardise and methodologies vary between centres. A joint working group, founded by the European Medical Research Council and the ESPGAN, have been trying to

develop an international protocol using standardised robust serological methods, positive and negative standard sera and reference material (Stern et al. 1996). Interestingly, comparison of the serological findings from 8 European laboratories showed more variation in the results of the objective AGA ELISA than the subjective IgA EmA test which showed almost 100% concordance for all centres (Stern et al. 1996). One would anticipate the same finding with the IgA anti-tTG ELISA. Standardisation of methodologies would reduce the wide variations of reported sensitivities and specificities, and permit more direct comparison of findings between different centres. A cost analysis in our lab has also shown that the IgA anti-tTG antibody ELISA is more expensive than the IgA EmA assay, with materials and analysis costing about £25 and £12 per sample respectively. The IgA and IgG AGA each cost about £12 per sample. An earlier report stating that the IgA anti-tTG ELISA is less expensive and easier to standardise (Trier, 1998) cannot be supported from my research.

The current in-house IgA anti-tTG ELISA cannot replace the IgA EmA assay for routine serological testing. Future development of the in-house assay should further assess the performance of the assay using calcium and excluding BSA. The assay performance may benefit most however, from a human recombinant tTG when it becomes commercially available. Further work should aim to modify the assay through collaboration with other groups, so that the assay methodology can be standardised and allow a more valid comparison of results.

9.3 Summary of Results

The performance of the serum and WGLF IgA anti-tTG antibody ELISA in the diagnosis and monitoring of CD patients, and the relationship between serology with genetics, histology and intestinal permeability were investigated. The main findings of this study were that IgA anti-tTG antibodies could be measured in serum and WGLF.

Serum antibody concentrations were significantly higher in untreated CD and DH patients than in treated CD, disease controls or healthy volunteers. In 100 untreated CD patients with normal serum total IgA, the sensitivity and specificity of IgA anti-tTG antibodies and IgA EmA were 78% and 97%, and 86% and 100% respectively. The PPVs were comparable at 98% and 100% respectively, but the NPV of IgA EmA (82%) was better than that of the IgA anti-tTG antibodies (74%). The IgG AGA was more sensitive but less specific than the IgA AGA (87% and 78% versus 60% and 86% respectively). The diagnosis of CD was improved by using a combination of serological tests. The combinations of IgA anti-tTG antibodies and IgA EmA with IgA and IgG AGA improved the overall sensitivities to 100% and 96% respectively, but at the cost of a reduction in the PPVs. The serum IgA anti-tTG antibody concentrations were also shown to progressively decline in CD patients following gluten withdrawal. It would have been useful to compare this decline in antibody concentrations with histology, but this was not possible as it was not routine practice to perform repeat biopsies following dietary treatment in all CD patients. The serum IgA anti-tTG antibody concentrations correlated well with matched serum IgA EmA titres, but like others, we did find some discrepancies.

Unexpected findings were the high prevalence of 25% for IgA EmA negative untreated CD patients and the low frequency of 61% for the DQ2 heterodimer ($\alpha 1^*0501$, $\beta 1^*0201$ heterodimer). The EmA negative patients did not have a significantly higher prevalence of autoimmune diseases, and most of these patients responded clinically and histologically to gluten withdrawal. The patients recruited into this study underwent intestinal biopsy on the basis of clinical presentation and not exclusively following the finding of positive serological results. There has been much reluctance from editing committees to believe that the IgA EmA negative patients with villous atrophy, on a normal gluten-containing diet and with normal serum total IgA concentrations, had CD. This bias was likely to be based on earlier studies showing nearly 100% sensitivity and

specificity of IgA EmA (Kumar et al. 1989; Whelan et al. 1996; Ferreira et al. 1992). It has since been noted that assay performance is greatly influenced by the selection of predominantly sero-positive patients for undergoing intestinal biopsy (Murray, 1999), and by the preferential evaluation of these tests in subjects with severe mucosal lesions (Rossi et al. 1988; Rostami et al. 1998). Indeed, our findings are supported by other studies which have recently found a comparable IgA EmA sensitivity of 74-78% (Valdimarsson et al. 1996; Rostami et al. 1999a; Dickey et al. 2000), indicating that our results were not attributable to poor methodology. It is important to highlight that IgA EmA negative CD patients exist so that intestinal biopsy is not delayed in symptomatic patients.

The WGL technique provided an ideal method for studying the secretion of locally produced antibodies into the gut lumen. The concentration of IgA anti-tTG antibodies and IgA AGA in WGLF was significantly higher in untreated CD patients than in disease or healthy controls. The higher WGLF IgA anti-tTG antibody concentrations in CD than controls is likely to be because jejunal mucosal TG activity is known to be raised in untreated CD, but is reduced in healthy subjects (Bruce et al. 1985) and in active colitis (D'Argenio et al. 1992). It would have been useful to study the change in antibody concentrations after gluten withdrawal, but patients were understandably reluctant to undergo the WGL procedure again for research purposes. We also showed that the WGLF IgA anti-tTG antibody concentrations compared well with WGLF total IgA and WGLF IgA EmA.

The measurement of IgA anti-tTG antibodies in WGLF was more sensitive than in serum (83% versus 72% respectively). The ROC curves showed that the WGLF assay was superior in performance to the serum assay. The lack of a correlation between serum and WGLF IgA anti-tTG antibodies and between WGLF IgA anti-tTG antibodies and WGLF total IgG concentrations suggested that intra-luminal antibodies were not

simply secreted from the serum into the gut lumen. This finding was not surprising as circulating IgA and lamina propria IgA are not thought to contribute significantly to the respective IgA pools (Conley and Delacroix, 1987). A raised WGLF total IgG was predominantly seen in IBD patients and was not typical of untreated CD. It was present however, in 4 CD patients indicating a protein losing enteropathy. The duodenal mucosal histology in 3/4 of these patients showed STVA, and the other patient with only mild PVA had collagenous colitis. The WGLF IgA anti-tTG antibody concentrations were significantly raised in Crohn's disease patients with active than inactive disease, but this was not observed in UC patients. This may be because of similarities in the immune mechanisms in CD and Crohn's disease. There was however, no association between the WGLF IgA anti-tTG antibody concentration and the anatomical distribution of Crohn's disease.

Our finding that WGLF, but not serum, IgA anti-tTG antibody concentrations were significantly higher in DQ2 positive than DQ2 negative CD patients, is consistent with theory that DQ2 presentation is favoured by gut-derived, but not peripheral, gliadin-specific T cells (Molberg et al. 1998). The DQ2 positive CD patients in this study however, did not have more severe histological lesions. There was also a small positive correlation between serum IgA anti-tTG antibody concentration and crypt depth suggesting a possible role for IgA anti-tTG antibodies in the process of crypt hyperplasia. This may have been because of the interaction between tTG and TGF- β as previously described. There was however no association between IgA anti-tTG antibody concentrations in serum or WGLF with IEL, CD3 or δ/γ TCR counts, morphometric measurements or intestinal permeability. It is possible that an association between serology and histology may have been found had there been more Marsh grades 1 and 4 lesions.

9.4 Advances in the Pathogenesis of Coeliac Disease

Since starting this project, there has been much progress in the understanding of the pathogenesis of CD. This progress has been based on the foundation acquired over the years starting with the identification that the protein fraction of wheat, gliadin, produces the mucosal injury and malabsorption in CD. The disproportionate abundance of glutamine (43%) compared to other amino acids, led to the concentration of the harmful effect of gliadin to be based on its abnormally high glutamine content (van de Kamer and Weijers, 1955). This group also showed that free glutamine and acid-deamidated gliadin were not toxic. It had been known for some time that tTG favoured binding to protein-bound glutamine residues (Folk, 1972), and it was later demonstrated that gliadin was an excellent substrate for tTG (Bruce et al. 1985). The jejunal mucosal tTG activity was shown to be significantly higher in untreated CD patients than in controls, and activity reduced after gluten withdrawal (Bruce et al. 1985).

Further convincing evidence of a role of tTG in the pathogenesis of CD came when tTG was shown to produce deamidation of specific glutamine residues in gliadin, and that this enhanced DQ2 binding and increased gut-associated T cell responsiveness (Molberg et al. 1998). It is still unknown if the involvement of tTG in the pathogenesis of CD is through disease initiation or alternatively, occurs as a secondary event following the T cell response to gluten and tissue damage (van de Wal et al. 2000). In the latter scenario, the release of cytosolic tTG would lead to the activation of gliadin-specific T cells, which help tTG-specific B cells in the formation of anti-tTG antibodies.

The role of antibodies in the pathogenesis of CD is still uncertain. CD is not a classical autoimmune disease, since IgA anti-tTG antibodies disappear following strict gluten withdrawal and the mucosal damage is reversed without residual fibrosis or scarring (Dieterich et al. 1997). The presence of gluten-induced antibodies, such as anti-tTG antibodies, in serum and WGLF may only be a useful epiphenomenon which help in the

diagnosis of CD, but do not contribute directly to the mucosal injury (Mowat, 1998). Compelling recent evidence of a direct contribution of anti-tTG antibodies to disease pathogenesis was the finding that serum IgA anti-tTG antibodies inhibited the differentiation of human epithelial cells and may directly contribute to the development of mucosal atrophy (Halttunen and Maki, 1999). The findings in this thesis support that the main pathogenic roles of tTG in CD are via its association with the DQ2 haplotype and its role in epithelial differentiation.

9.5 Future Strategies for the Treatment of Coeliac Disease

Dietary gluten withdrawal has been the mainstay in the therapy of CD. The finding that tTG is the autoantigen in CD has also led to the novel theories for the potential treatment of CD. Firstly, it has been proposed that oral feeding of tTG may induce oral tolerance (Dieterich et al. 1997). Since oral tolerance operates at the level of the T cell, then the ingestion of tTG in theory would lead to the “silencing” of tTG-specific T cells (Sollid et al. 1997). The convincing argument from Sollid *et al.* that gliadin-specific, and not tTG-specific, T cells are involved explains why anti-tTG antibody concentrations decline after gluten is withdrawn from the diet (Sollid et al. 1997). If tTG-specific T cells were involved, then the anti-tTG antibody levels would not be affected by diet. The confirmation that tTG and gliadin may be cross-linked (Molberg et al. 1998) supports the theory that tTG-specific T cells are not required to induce an immune response to tTG. Therefore, oral feeding of tTG is unlikely to represent a successful future therapy.

Current work on identifying disease eliciting peptides may offer a new possibility for CD prevention and treatment. It has been suggested that wheat deficient in T cell stimulatory peptides can be constructed (van de Wal et al. 2000). As the T cell response appears to be directed to multiple peptides, and the peptide sequences may be

repetitive, it will be extremely difficult to construct non-toxic wheat strains. Hope for the future development of a “non-toxic” wheat has re-surfaced following the recent report that tTG specifically deamidates Q65 in the A-gliadin peptide (amino acids 56-75) (Anderson et al. 2000). Removal or modification of this specific immunodominant epitope, or therapy with an “altered peptide ligand antagonist” has been suggested immunomodulatory strategies. Although the prospect of manipulating disease eliciting peptides which hold the potential to prevent disease induction is attractive, in the current climate with fears about the safety of genetically modified food, this approach may not be acceptable to some patients.

Finally, interference with tTG activity in the intestine has been proposed as a possible therapeutic strategy (van de Wal et al. 2000). Blocking tTG activity however, may have serious consequences as the enzyme is involved in regulating physiological mechanisms, including tissue damage, in the gut. Alternatively, attempts can be made to prevent the process of tTG-mediated deamidation, which as far as I am aware, serves no physiological function. As explained earlier, tTG cross-links protein-bound glutamine and lysine residues. In the absence of lysine residues however, deamidation occurs where the enzyme catalyses hydrolysis at the carboxamide group of peptide-bound glutamine to give peptide-bound glutamic acid and ammonia. It has not been demonstrated if deamidation would still occur *in vivo* if lysine residues were available. I would suggest that deamidation, and hence the T cell activation, could in theory therefore be prevented by the provision of protein-bound lysine residues which would promote a cross-linking reaction. These theories are still some way from offering an alternative therapy for CD patients, and I believe that my theory would warrant future consideration.

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APPENDIX

Serum IgA tissue transglutaminase antibodies in coeliac disease and other gastrointestinal diseases

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Summary

We investigated the presence of IgA anti-tissue transglutaminase (tTG) antibodies in untreated coeliac disease (CD) and other gastrointestinal diseases, and compared IgA tTG concentrations with anti-endomysium (EMA) immunofluorescent findings. The study included 116 untreated CD patients (74 female, 42 male, age range 15–78 years, median 47 years), 82 treated CD patients, 65 patients with normal duodenal histology, 260 disease control samples and 29 healthy volunteers. IgA anti-tTG, EMA, and anti-gliadin (AGA) antibodies were measured. Serum total IgA was measured in the CD patients. Two IgA-deficient untreated CD patients were excluded. IgA EMA

and IgA AGA were positive in 99 (87%) and 69 (61%), respectively, of the 114 untreated CD patients. Elevated IgA anti-tTG were found in 92/114 (81%) untreated coeliacs, 1/82 (1%) treated coeliacs, 2/65 (3%) non-coeliacs, 10/260 (4%) disease controls and 2/29 (7%) volunteers. Four of the untreated CD patients, with a normal serum total IgA concentration, were negative for all the serological tests. IgA anti-tTG concentrations were significantly higher in untreated coeliacs (median 10 200 units/ml) than in other groups (Mann–Whitney, $p < 0.00001$) and compared well with IgA EMA titres ($r^2 = 0.54$; $p < 0.0001$).

Introduction

Serological testing was included in the 1989 revision of the European Society of Paediatric Gastroenterology and Nutrition criteria for the diagnosis of coeliac disease (CD).¹ Problems with the current serological methods include a lack of standardized methodologies, differing diagnostic practices and the varying populations studied. The sensitivities/specificities of IgA anti-gliadin antibodies (AGA) and IgA anti-endomysium antibodies (EMA) vary widely: 46–100%/86–100% for AGA,^{2–4} and 74–100%/91–100%^{5–8} for EMA. The recent finding that the enzyme tissue transglutaminase (tTG) is the auto-antigen that interacts with EMA antibodies in CD sera⁹ has led to the development of an enzyme-linked immunosorbent assay (ELISA). In theory, a semi-quantitative, objective ELISA would

be superior to the labour-intensive, subjective, immunofluorescent EMA technique for population screening.

tTG is a calcium-dependent enzyme that catalyses the formation of ϵ -(γ -glutamyl) lysine bonds between protein-bound glutamine and lysine residues, and is widely distributed in body fluids and tissues¹⁰ including the gastrointestinal sub-mucosa.^{11,12} Through its cross-linking activity, tTG has been implicated in many physiological processes, including wound healing, via its interaction with extracellular matrix proteins.¹³ Gliadin is an excellent substrate for the enzyme, as it consists of about 40% glutamine residues,¹⁴ a necessary prerequisite for the enzyme.¹⁰ In CD, tTG activity is increased in the jejunal mucosa.¹⁴ tTG provides a

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plausible mechanism for gliadin toxicity through its selective deamidation of glutamine residues of gliadin, creating epitopes which favour gliadin recognition by gut-derived T cells when presented by DQ2 molecules.¹⁵

The performance of the IgA anti-tTG ELISA has previously been reported in untreated CD patients with predominantly positive IgA EMA antibodies.^{16–19} This study differs from other recent reports in evaluating the IgA anti-tTG ELISA in diagnosing untreated CD patients, including a large proportion of IgA-EMA-negative CD patients. We have also compared the sensitivity of IgA anti-tTG to the conventionally used IgA EMA and IgA AGA tests. As tTG is widely distributed in the gut, we have also investigated its disease specificity by analysing sera from patients with a wide range of gastrointestinal disorders, including inflammatory, infectious, functional and neoplastic aetiologies. In addition, we address the issue of calcium activation of the enzyme on the assay characteristics, as there have been conflicting reports in recent studies, with some reporting calcium to be essential,^{16,17} while others disagree.^{18,20}

Methods

Patients

All patients recruited presented to the Gastroenterology Unit at the Western General Hospital in Edinburgh. Small-bowel mucosal biopsies were performed on clinical suspicion, and not on the basis of positive serology alone. Clinical presentations for which intestinal biopsy was performed included the investigation of diarrhoea, weight loss, anaemia, or the presence of an associated disorder (e.g. dermatitis herpetiformis or insulin-dependent diabetes mellitus). A total of 658 samples from 643 individuals were analysed (Table 1). Ethical

approval was obtained from the Medicine and Oncology Subcommittee of Lothian Ethics in Research Committee.

Untreated CD patients

Serum samples were studied from 116 untreated CD patients (74 females, 42 males; age range 15–78 years, median 47 years) diagnosed between 1988 and 1999. Small-intestinal biopsies were obtained from all patients. Serum samples stored at -70°C were available for all patients while on a normal gluten-containing diet and in 32 patients following gluten withdrawal (range 2–12 months, median 5 months). All untreated CD patients had characteristic histological findings and responded clinically to gluten withdrawal. IgA-EMA-negative CD patients were offered a repeat biopsy following treatment with a gluten-free diet (GFD) to support the diagnosis of CD.

Treated CD patients

Serum samples were analysed from 82 treated CD patients (62 females, 20 males; age range 15–82 years, median 53 years) attending the GI unit coeliac clinic. Dietary compliance was based on a dietary history and a negative IgA EMA antibody result.

Non-coeliac controls

Sera were obtained from 65 patients (45 females, 20 males; age range 16–90 years, median 45 years) investigated for suspected CD, but who subsequently had normal duodenal mucosal histology.

Disease controls

A total of 260 serum samples were analysed from 245 patients undergoing GI investigations. The diagnoses of these patients were Crohn's disease (68), ulcerative colitis (80), irritable bowel syndrome (36), infective or idiopathic diarrhoea (15)

Table 1 Disease categories and patient demographics

Disease category	Samples (n)	Patients (n)	Gender (F/M)	Age range (years)	Median age (years)
Untreated coeliacs	116	116	74/42	15–78	47
Treated coeliacs	82	82	62/20	15–82	53
Non-coeliac controls	65	65	45/20	16–90	45
Crohn's disease	81	68	41/27	17–82	41
Ulcerative colitis	82	80	34/46	15–80	45
IBS	36	36	25/11	16–91	39
IID	15	15	9/6	22–78	51
Other intestinal diseases	46	46	28/18	19–82	54
Healthy volunteers	29	29	19/10	21–58	35
Anonymous blood donors	106	106	Unknown	Unknown	Unknown

IBS, irritable bowel syndrome; IID, infective/idiopathic diarrhoea.

and miscellaneous other diagnoses including gastrointestinal malignancy and liver diseases (46). Small-bowel mucosal biopsies were not available for all of these patients.

Healthy controls

Serum samples were prospectively analysed from 29 healthy volunteers and 106 anonymous blood donors.

Laboratory methods

All serum samples were analysed for IgA-class AGA, EMA and anti-tTG antibodies. IgG AGA analyses were done in all of the untreated CD and non-CD control sera. IgA EMA antibodies were titrated to the end-point in 38 of the untreated CD patients. Serum total IgA was measured in all untreated CD patients. ELISA methods for determining AGA and total IgA were as previously described.²¹ The upper reference limits of IgA and IgG AGA were ≥ 30 units/ml and ≥ 45 units/ml, respectively.

IgA anti-endomysium antibodies

Cryostat sections of human umbilical cord (8 microns thick) were cut and stored at -20°C . Frozen sections were thawed at room temperature for 30 min, fixed in acetone for 10 min at -20°C , and then transferred to chloroform for 30 min at room temperature. Each section was covered with diluent (0.9% sodium chloride, 0.05% Tween 20 and 1% adult bovine serum) for 10 min and then drained. Serum (50 μl) (diluted at 1/5) was added to each section and incubated at room temperature for 60 min. Each batch included a positive and a negative serum control.

Sections were rinsed in PBS, then FITC-conjugated sheep anti-human IgA (Scottish Antibody Production Unit, Product No. S073-201) (diluted at 1/20 in ELISA diluent) was added to each section, and they were incubated for 60 min in the dark. Slides were placed into a dish of PBS, rinsed with running water for 5 min, dried and then mounted in glycerin. The slides were examined by two independent readers at $\times 10$ and $\times 25$ magnifications using an immunofluorescence microscope (H500, Hund, Wetzlar), and a positive result was recorded if the connective tissue surrounding the muscle cells fluoresced brightly in a honeycomb pattern. The EMA was reported as equivocal if the readers could not confidently interpret the immunofluorescent pattern; this result did not represent disagreements between readers. The equivocal results have been grouped with the EMA-negative results for analysis in this paper. The two experienced readers were both blinded as

to the final diagnoses and clinical details of the patients to avoid bias in this subjective assessment.

IgA anti-tissue transglutaminase antibodies

Immulon 2 (Dynatech) plates were coated with 100 ml/well of 10 mg/ml solution of guinea pig liver transglutaminase (Sigma, T5398) diluted in a carbonate-bicarbonate-coating buffer (0.05 M, pH 9.6) and incubated overnight in a covered moist box at 4°C . Plates were washed three times with wash solution (0.9% saline, 0.05% Tween 20) and blocked with 250 ml/well of blocking solution (phosphate-buffered saline (PBS), 1% bovine serum albumin and 0.05% Tween 20) at room temperature for 2 h.

Serum from an untreated CD patient with known high anti-tTG antibody levels was used as a standard. The standard material was doubly diluted in duplicate from 1/50. High and low serum quality controls, and test serum samples were diluted in duplicate at 1/50 and 1/100 in a diluent of PBS with 0.05% Tween 20. Plates were incubated overnight at 4°C and then washed three times in wash solution. Goat anti-human IgA (α -chain-specific) alkaline phosphatase conjugate, 100 ml/well, diluted at 1/800, was incubated at room temperature for 5 h and then washed three times with wash solution.

A p-nitrophenylphosphate substrate in diethanolamine buffer (Sigma) was added, and colour development was monitored by measuring A_{405} on an ELISA reader. The absorbance endpoint for IgA anti-tTG was taken when the top standard dilution reached an optical density of 1.0. Test results were technically acceptable if the quality control IgA anti-tTG concentrations were within ± 2 SD of the mean of values obtained from previous runs, and the duplicate values differed by no more than 10%. The inter-assay and intra-assay coefficients of variation were 9.1% and 11.7%, respectively. The serum reference standard was designated as 25 000 units/ml and the lower limit of detection in serum was 300 units/ml. The normal upper reference range for the IgA anti-tTG ELISA of 2950 units/ml was calculated using a non-parametric percentile method²² which excluded 95% of the 106 anonymous blood donors.

Effect of calcium and pH during the coating stage of the tTG ELISA

To investigate the requirement for calcium during the coating stage, we added 5 mM calcium chloride (Sigma) to the carbonate-bicarbonate coating buffer (pH 9.6) and compared this to a Tris/HCl system (pH 7.5) with 5 mM calcium chloride. Doubling dilutions of three serum samples were performed and A_{405} readings were recorded. BSA in the

blocking solution was substituted with haemoglobin at 1 mg/ml to exclude possible interaction of antibodies with BSA.

Statistical analysis

The sensitivity of the IgA anti-tTG ELISA was calculated as the frequency of positive results in biopsy-proven CD patients while on a normal diet. The specificity of the assay was calculated from the group of non-CD controls found to have normal duodenal histology, and was not based on the disease controls from whom an intestinal biopsy was not obtained. The Mann-Whitney test was used to compare IgA anti-tTG antibody concentrations in untreated CD patients and the disease control groups. Pearson's correlation was applied to determine the correlation between serum IgA anti-tTG and the corresponding IgA EMA titre, and the significance was determined by linear regression.

Results

Effect of calcium during the coating stage of the tTG ELISA

The addition of 5 mM calcium chloride to the carbonate-bicarbonate buffer (pH 9.6) resulted in a reduction of optical density readings in two of the three samples studied. The optical densities recorded using the carbonate-bicarbonate system without calcium were more comparable to the Tris/HCl coating method (pH of 7.5) with 5 mmol/l of calcium than following the addition of calcium to our system (Figure 1).

Serum total IgA in untreated coeliac disease patients

Two of the 116 untreated CD patients had selective IgA deficiency (total serum IgA <0.07 mg/l). Duodenal histology had improved markedly in both of these patients following treatment with a GFD. Serum IgA-class anti-tTG antibodies, AGA and EMA were not detectable. IgG AGA was raised in both patients. IgG anti-tTG antibodies were not measured. IgA-class serology results from these two male patients have been excluded from the calculations of assay sensitivity ($n = 114$).

Serum IgA anti-tTG antibody and IgA EMA

Untreated coeliac disease patients

IgA anti-tTG antibodies were raised in 81% (92/114) of the untreated CD patients and 1%

(1/82) of the treated CD patients (Table 2). Serum IgA anti-tTG antibody concentrations were significantly higher in untreated CD patients (median 10 200 units/ml; range 300–89 400 units/ml) than in all other control groups (Mann-Whitney test, $p < 0.0001$) (Figure 2). Of the 22 untreated CD patients with normal serum IgA anti-tTG and total IgA concentrations, 11 were positive for IgA EMA, six for IgA AGA and 14 for IgG AGA (Table 3). Three untreated CD patients had borderline negative IgA anti-tTG concentrations of 2220 units/ml, 2340 units/ml and 2510 units/ml, respectively.

IgA EMA was positive in 99/114 (87%) of untreated CD patients, negative in 14/114 (12%) and equivocal in 1/114 (1%). The IgA anti-tTG antibody concentration was raised in four of these patients. The IgA AGA and IgG AGA were raised in three and 11 of these patients, respectively. Repeat biopsy following treatment with a GFD was done in 12/15 EMA-negative CD patients. Duodenal histology had improved in nine, but remained unchanged in four, of whom two were non-compliant with their diet. The two patients who did not have repeat duodenal biopsies both had a clinical response to gluten withdrawal.

The sensitivity of the IgA anti-tTG ELISA would increase from 81% (92/114) to 89% (88/99) if only the IgA-EMA-positive patients were included. Using a combination of tests could further enhance the serological detection of untreated CD (Table 4). The highest sensitivities in diagnosing CD of 93% and 96% resulted from the combination with IgG AGA of either IgA anti-tTG or IgA EMA, respectively. The use of IgG AGA as a screening test, however, produces a false-positive rate in controls of 22% (14/65). The combination of IgA anti-tTG or IgA EMA with IgA AGA produced sensitivities of 86% and 89%, respectively, with a lower false-positive detection rate for IgA AGA of 9/65 (14%).

Control patients

Elevated IgA anti-tTG results were found in 1/82 (1%) of treated CD patients, in 2/65 (3%) of non-CD controls, in 10/260 (4%) of gastrointestinal disease controls and in 2/29 (7%) of healthy volunteers (Table 2). Of the treated CD patients, 14/82 (17%) remained IgA AGA-positive. One of the two non-CD controls with an elevated IgA anti-tTG concentration of 29 000 units/ml, but negative IgA EMA, was being investigated for diarrhoea and abdominal cramps and had a normal duodenal biopsy. In retrospect, this lady admitted to consuming a low gluten diet at the time of her intestinal biopsy and serum sampling. This patient declined a supervised gluten challenge and repeat small-intestinal biopsy. The other non-CD control with a raised IgA anti-tTG concentration of 4860 units/ml,

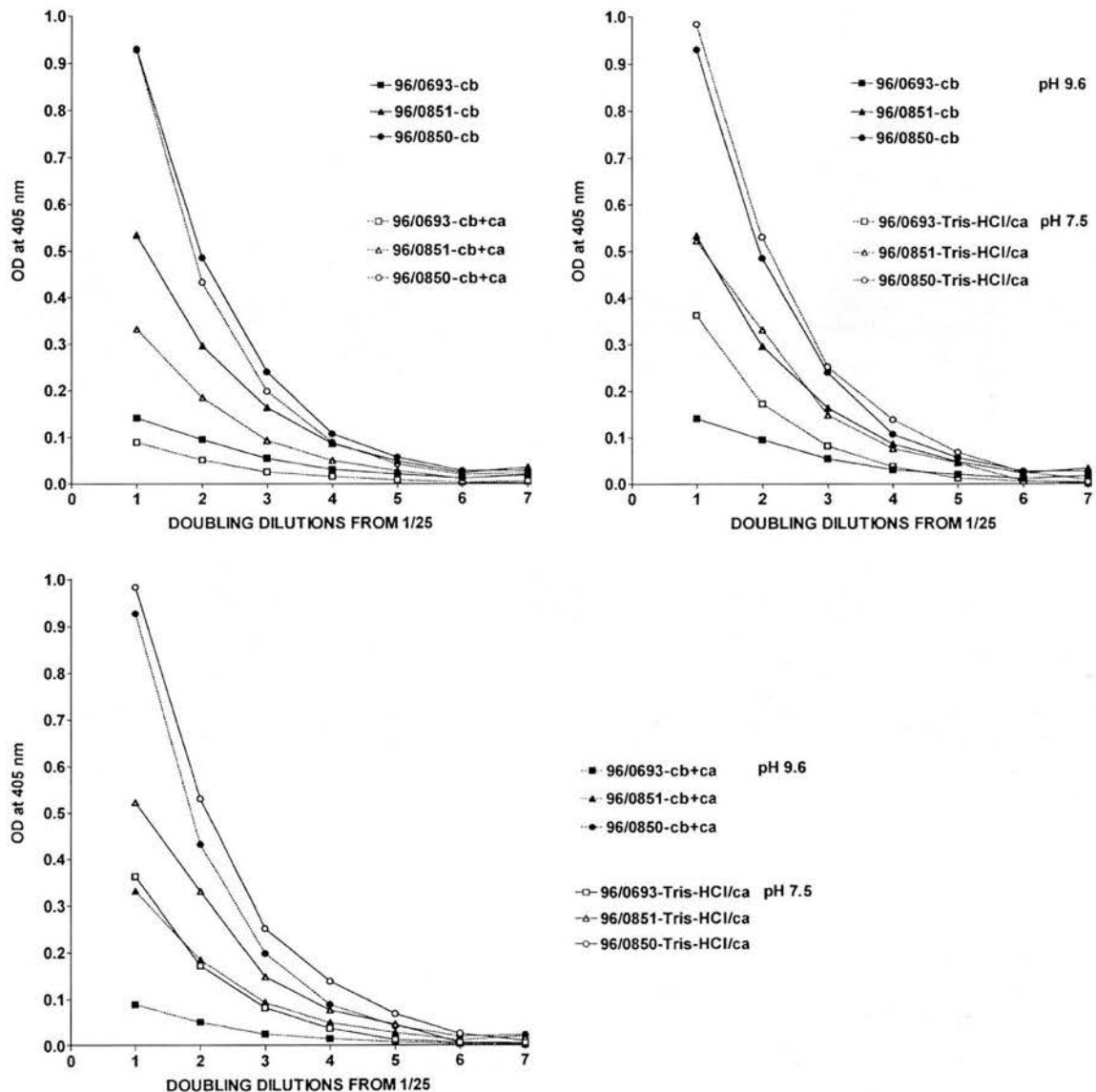


Figure 1. The effect of calcium on the optical density readings of the IgA anti-tTG ELISA using the carbonate-bicarbonate and the Tris/HCl coating buffer systems. Calcium added to the carbonate-bicarbonate system reduces the optical densities.

Table 2 Serological results in untreated coeliac disease and control patients

Disease category	IgA AGA +ve	IgA EMA +ve	IgA tTG +ve	Median IgA tTG (units/ml)
Untreated coeliacs	69/114 (61%)	99/114 (87%)	92/114 (81%)	10200
Treated coeliacs	14/82 (17%)	0/82	1/82 (1%)	635
Non-coeliac controls	9/65 (14%)	0/65	2/65 (3%)	300
Crohn's disease	13/81 (16%)	1/81 (1%)	2/81 (2%)	880
Ulcerative colitis	13/82 (16%)	0/82	2/82 (2%)	725
IBS	2/36 (6%)	0/36	1/36 (3%)	560
IID	3/15 (20%)	0/15	2/15 ^a (13%)	1000
Other intestinal diseases	8/46 (17%)	0/46	3/46 ^b (7%)	760
Healthy volunteers	0/29	1/29 (3%)	2/29 (7%)	410

Two untreated CD patients with selective IgA deficiency have been excluded from the assay sensitivity calculations. Five patients indicated by the symbols, ^a and ^b had elevated IgA anti-tTG antibodies. The diagnoses of these patients were giardiasis, malabsorption following intestinal bypass surgery for obesity, rectal tubular adenoma, pneumatosis cystoides intestinalis and Cronkhite-Canada syndrome. IBS, irritable bowel syndrome; IID, infective/idiopathic diarrhoea.

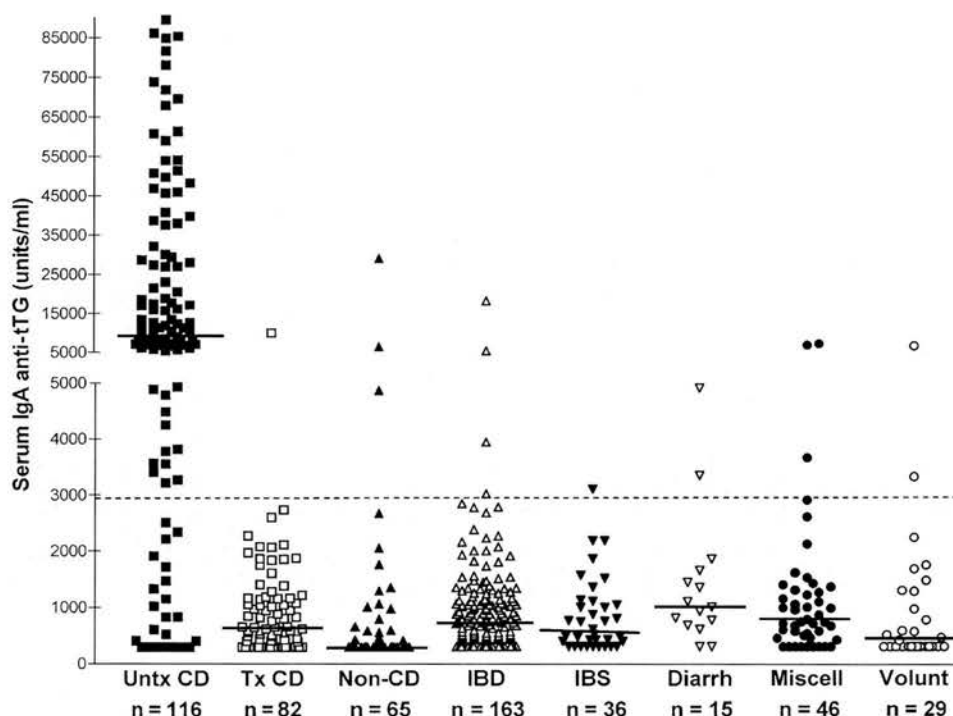


Figure 2. Serum IgA anti-tTG antibodies in patients with untreated CD (Untx CD; $n = 116$), treated CD (Tx CD; $n = 82$), biopsy-proven non-CD (Non-CD; $n = 65$), inflammatory bowel disease (IBD; $n = 163$), irritable bowel syndrome (IBS; $n = 36$), idiopathic or infective diarrhoea (Diarrh; $n = 15$), miscellaneous other gastrointestinal disorders (Miscell.; $n = 46$), healthy volunteers (Volunt; $n = 29$). The dotted horizontal indicates the upper reference limit and the median values are indicated. Serum IgA anti-tTG is significantly higher in untreated CD patients than in all other groups (Mann-Whitney test $p < 0.0001$).

was a 40-year-old lady who presented with symptoms of abdominal discomfort, loose stools alternating with constipation and dyspepsia. An upper endoscopy with duodenal histology and a barium enema were both normal. The IgA and IgG AGA were negative. Her symptoms were thought to be due to functional bowel disease.

One Crohn's disease patient with an IgA anti-tTG concentration of 5260 units/ml had a positive IgA EMA but a normal duodenal biopsy including intra-epithelial lymphocyte count. One patient with ulcerative colitis had a raised IgA anti-tTG concentration of 18100 units/ml but a negative IgA EMA and AGA. This individual has not undergone small-intestinal biopsy.

Two of the healthy male volunteers had elevated IgA anti-tTG concentrations of 6495 units/ml and 3315 units/ml, respectively. Only the former was EMA positive and subsequent duodenal biopsies showed partial villous atrophy consistent with untreated CD. In retrospect, this 'asymptomatic' member of the medical staff reported intermittent diarrhoea and dyspepsia. There was no family history of CD, but his sister is currently being investigated for anaemia. The other volunteer was IgA AGA- and EMA-negative and has not undergone duodenal biopsy.

Other serological results

Serum IgA and IgG AGA were raised in 61% (69/114) and 87% (99/114) of untreated CD patients, respectively, and in 14% (9/65) and 22% (14/65) of non-CD controls with normal duodenal biopsies, respectively. Elevated IgA AGA concentrations were also seen in 15% (39/260) of disease controls and 17% (14/82) of treated CD patients, but in none of the 29 healthy volunteers. Four untreated CD patients were negative for all serological markers studied.

IgA anti-tTG antibodies following treatment with a gluten-free diet

Of the 116 untreated CD patients, serum samples were obtained from 32 after treatment with a gluten free diet (GFD) for a median 5 months (range 2–12 months). IgA anti-tTG titres were elevated in 23/32 (72%) patients before commencing the GFD. IgA anti-tTG antibody concentrations remained negative following gluten withdrawal in nine (28%) patients and are not shown. IgA anti-tTG antibody concentrations reduced rapidly following GFD introduction (usually within the first 5 months), returning to normal in 18/23 (78%)

Table 3 Serological results of the CD patients with normal IgA anti-tTG antibody concentrations

Patient	Serum IgA anti-tTG (units/ml)	Serum IgA EMA	Serum IgA AGA (units/ml)	Serum IgG AGA (units/ml)
1	300	Positive	13	85
2	300	Positive	31	34
3	300	Negative	13	97
4	300	Negative	4	157
5	300	Negative	3	20
6	300	Negative	7	4
7	300	Negative	12	11
8	410	Positive	20	42
9	410	Negative	29	146
10	520	Negative	2	21
11	610	Negative	6	236
12	830	Positive	71	112
13	830	Negative	17	181
14	1020	Positive	5	123
15	1150	Negative	19	145
16	1330	Positive	67	216
17	1470	Positive	96	429
18	1720	Negative	7	10
19	1910	Positive	15	117
20	2220	Positive	74	178
21	2340	Positive	5	54
22	2510	Positive	139	40

Of the 114 untreated CD patients with normal serum total IgA concentrations, the IgA anti-tTG antibody concentrations were low in 22 (19%) patients. Of these 22 patients, 11 were IgA EMA-, six were IgA AGA- and 14 IgG AGA-positive. Three untreated CD patients had borderline negative IgA anti-tTG concentrations of 2220 units/ml, 2340 units/ml and 2510 units/ml.

Table 4 Sensitivity of serological markers when used in combination

Serological tests	Sensitivity	Serological tests	Sensitivity
IgA tTG	92/114 (81%)	IgA EMA	99/114 (87%)
IgA tTG + IgA AGA	98/114 (86%)	IgA EMA + IgA AGA	102/114 (89%)
IgA tTG + IgG AGA	106/114 (93%)	IgA EMA + IgG AGA	109/114 (96%)
IgA tTG + IgA AGA + IgG AGA	108/114 (95%)	IgA EMA + IgA AGA + IgG AGA	109/114 (96%)

Two untreated CD patients with selective IgA deficiency have been excluded from the assay sensitivity calculations. Combining serological methods increases the sensitivity of serological methods, especially when both IgA- and IgG-class antibodies are used.

of patients within 12 months (IgA anti-tTG concentrations pre-GFD: median 15 700 U/ml, range 4480–81 600 U/ml; post-GFD: median 1621 U/ml, range 613–7304 units/ml) (Figure 3). Two patients consented to attend regularly for close monitoring of IgA anti-tTG concentrations after starting of their GFD and the decline in their antibody concentrations is shown in Figure 4. IgA AGA and IgA EMA antibodies were positive in 22/32 (69%) and in 24/32 (75%) untreated CD patients, respectively. Of the 24 EMA-positive patients, 15 (47%) sero-reverted, compared to six (27%) of the 22 IgA AGA-positive patients during the study period.

Association between serum IgA anti-tTG and EMA antibodies

Serum IgA anti-tTG antibodies were significantly higher in the 101 EMA-positive (median 12 600 units/ml; range <300–89 400 U/ml) than in the 449 EMA-negative (median 620 units/ml; range 300–18 100 units/ml) serum samples (Mann–Whitney test, $p < 0.0001$) (Figure 5). The IgA anti-tTG concentrations in 37 IgA EMA-positive untreated CD patients correlated well with the IgA EMA titres ($r = 0.54$; $p < 0.0001$). The IgA anti-tTG was not significantly higher in patients with partial villous atrophy (PVA) compared to those with

increased intra-epithelial lymphocytes (IELs), or in those with subtotal villous atrophy (STVA) compared to those with PVA (Mann-Whitney, $p=0.3$ and $p=0.7$, respectively) (Figure 6).

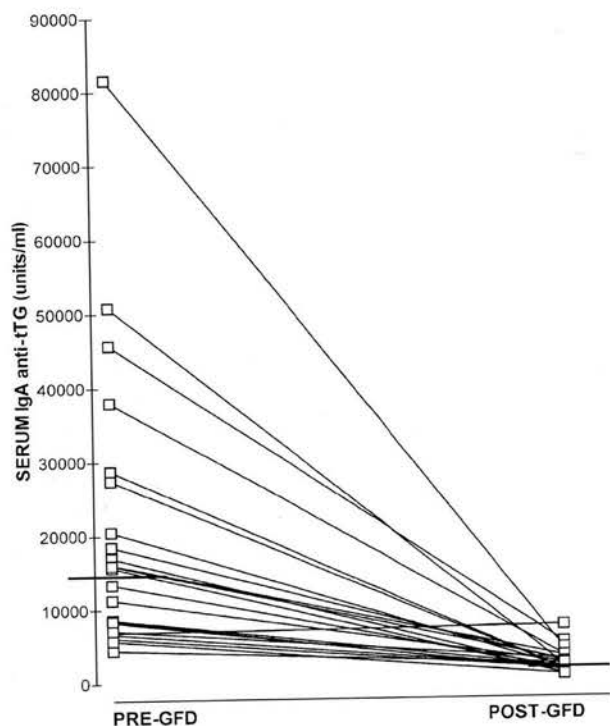


Figure 3. Serum IgA anti-tTG antibodies declined in all 23 untreated CD patients with initially elevated antibody levels following treatment with a gluten-free diet (GFD) for a median duration of 5 months (range 2–12 months).

Discussion

Serological testing using the IgA anti-tTG ELISA is useful, but is not superior to the IgA EMA immunofluorescent test in screening for CD. IgA anti-tTG concentrations are higher in untreated CD than in treated CD, other gastrointestinal diseases, or healthy controls. The sensitivities of the IgA anti-tTG ELISA and IgA EMA were comparable at 81% and 87%, respectively, and IgA anti-tTG concentrations and IgA EMA titres compared well. Our finding of positive IgA anti-tTG and EMA serology in a healthy 'asymptomatic' volunteer with partial villous atrophy supports the observation that subtle presentations of CD may easily be missed.^{23,24}

Past reports of assay sensitivity and specificity have been greatly influenced by performing intestinal biopsy on predominantly seropositive patients.²⁵ The CD patients in this study however, were biopsied on the basis of clinical suspicion, and were not selected exclusively by serology. This approach produced a high prevalence of EMA-negative untreated CD patients (15%, 17/116), of whom only two had selective IgA deficiency. The other subject had an equivocal EMA result. The high proportion of EMA-negative CD patients reduced the IgA anti-tTG ELISA sensitivity, as it improved from 81% to 89% when only EMA-positive CD patients were considered. The sensitivities of IgA anti-tTG and EMA improved when used in combination with IgG AGA, giving sensitivities of 93% and 96%, respectively. The addition

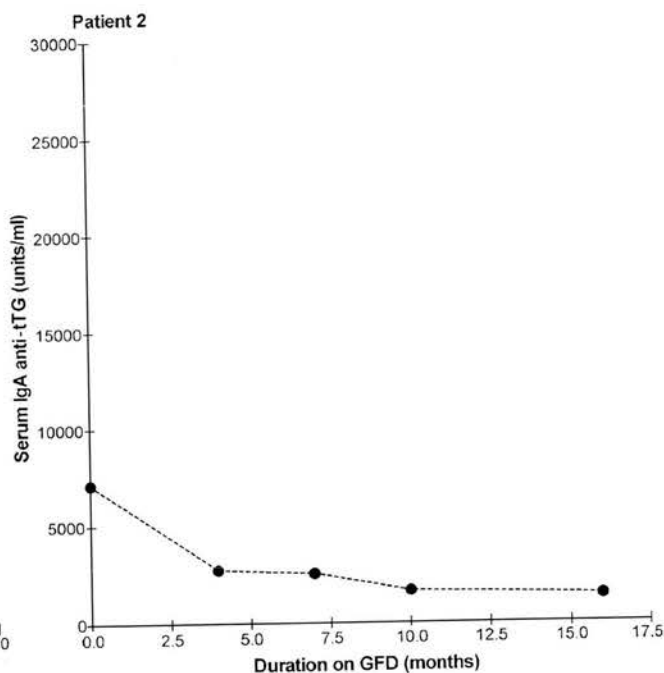
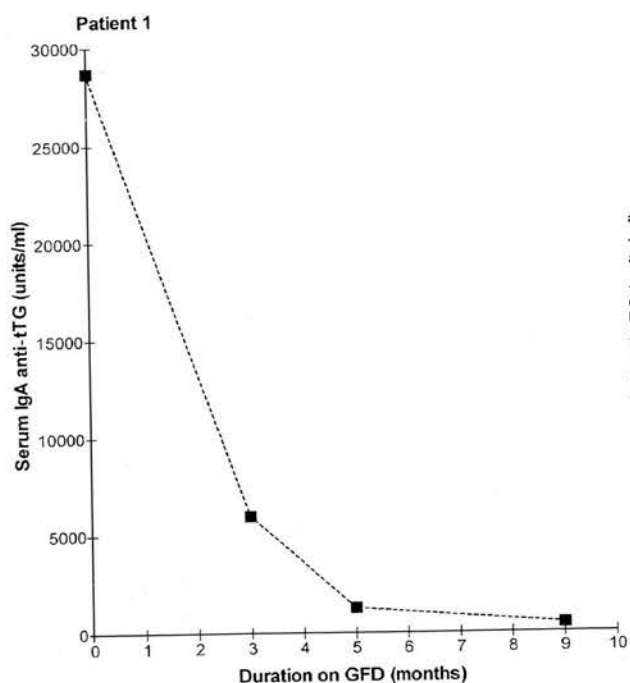


Figure 4. Serum IgA anti-tTG antibodies declined rapidly following treatment with a gluten-free diet (GFD) in two closely monitored CD patients.

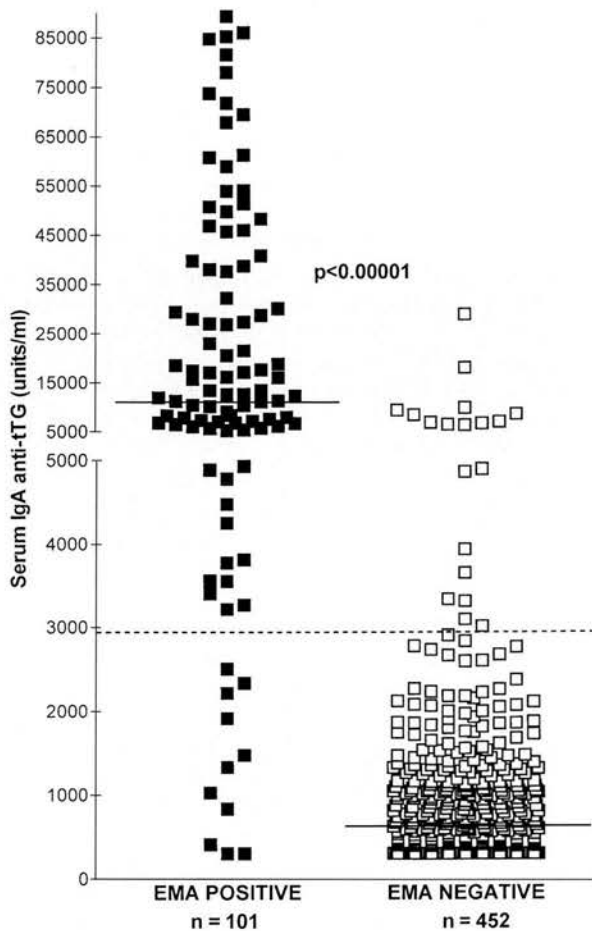


Figure 5. Serum IgA anti-tTG concentrations were significantly higher in EMA-positive ($n=101$) than in EMA-negative ($n=449$) samples (Mann-Whitney test: $p<0.00001$). Median values are shown.

of IgG AGA, however, resulted in a reduction in specificity.

The IgA anti-tTG ELISA positively identified four EMA-negative untreated CD patients, but failed to detect 11 EMA-positive patients. Others have also found discrepancies between IgA anti-tTG and EMA.¹⁷ We found positive IgA anti-tTG, IgA EMA and IgA AGA antibodies in 4% (10/260), 0.4% (1/260) and 15% (39/260) of our disease controls respectively. Sulkanen *et al.* did not find positive IgA anti-tTG antibodies in any of their 32 IBD patients, but did find positive results in other gastrointestinal controls.¹⁶ The finding of IgA anti-tTG antibodies in non-CD subjects may be explained by the wide distribution of the enzyme throughout the gastrointestinal tract.^{11,12} The lower specificity of the IgA anti-tTG ELISA than the IgA EMA may also be due to the use of a non-human, guinea pig liver as the source of tTG in comparison to the use of human umbilical cord, used in the EMA assay. The high IgA anti-tTG antibody titres found in untreated CD patients is probably due

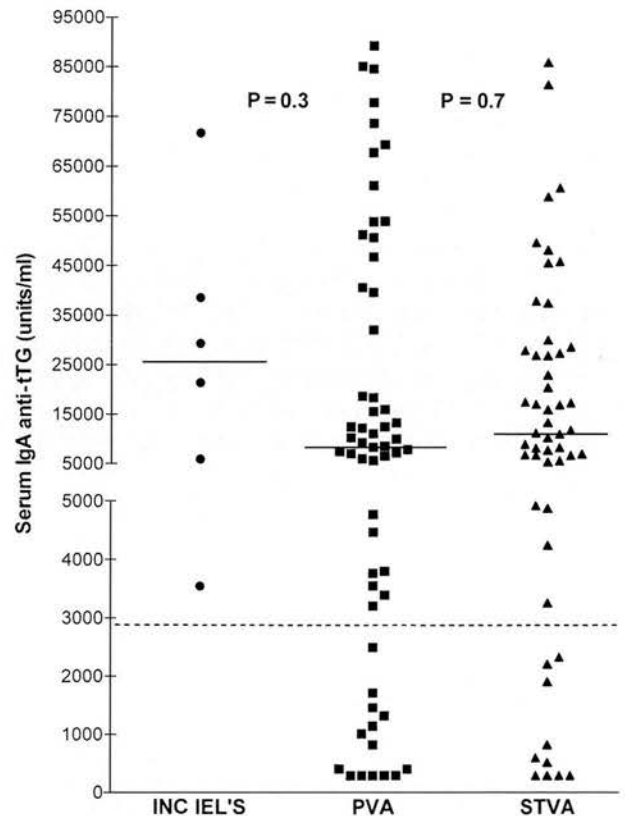


Figure 6. Serum IgA anti-tTG concentrations compared to duodenal mucosa histology in untreated CD patients. Serum IgA anti-tTG was not significantly higher in patients with sub-total villous atrophy (STVA) than in those with partial villous atrophy (PVA) (Mann-Whitney test: $p=0.7$).

to the suitability of dietary gliadin as a substrate for tTG, and because the concentration of gliadin is highest in the proximal intestine.

Recent reports on the sensitivities and specificities of the IgA anti-tTG ELISA (also using the guinea pig liver source) have ranged from 85% to 98.1% and from 94% to 98%, respectively.^{16-19,26} However, few of the untreated CD patients investigated in these studies were EMA-negative: 10/136,¹⁶ 1/106,¹⁷ 1/27,¹⁸ and 0/39.¹⁹ Rostami *et al.* found that the sensitivities of IgA anti-tTG²⁷ and IgA EMA²⁸ correlated closely with histological severity. EMA positivity has also been thought to depend on the length of intestine involved,²⁹ as well as on genetic factors.³⁰ However, we did not find an association between antibody titres and histological severity.

The requirement for calcium activation of the enzyme has been disputed. In our hands, the enhanced sensitivity of calcium activation appears to be pH-dependent, as in our assay (pH 9.6), the optical density readings fell sharply following the addition of calcium. The finding that calcium is not essential is in agreement with recent reports.^{18,20}

Recently, a radio-binding assay using human recombinant tTG has been shown to compare well with EMA findings, with a sensitivity of 99.1% (111/112) and specificity of 95.7%.³¹ This technique was superior to the IgA anti-tTG ELISA using guinea pig liver tTG dissolved in PBS at pH 7.3 (also without calcium and blocked with BSA), which had a sensitivity of 85% and specificity of 91% in the same patient group.³¹ These latter findings compare well with our results in EMA-positive patients using a similar method except for the coating pH. Human recombinant tTG would appear to be better than the guinea pig liver source, which only shares 80% sequence homology with the human enzyme. Both the sensitivity and specificity of the IgA anti-tTG ELISA may improve in the future when a human recombinant tTG becomes commercially available.

Acknowledgements

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Anti-endomysial Antibody Negative Celiac Disease

Does Additional Serological Testing Help?

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Anti-endomysium antibodies (AEM) fail to identify all untreated celiac disease (CD) patients. This study aims to determine if additional serology, in particular, IgA anti-tissue transglutaminase (tTG) antibodies, increases detection. Fifty-three biopsy-proven untreated CD patients (39 women, 14 men; median age 51 years) and 65 control patients with normal duodenal histology (46 women, 19 men; age range 17–90 years, median 45 years) were prospectively studied. Serum total IgA, IgA anti-tTG, IgA AEM, IgA anti-gliadin (AGA) and IgG AGA antibodies were measured. Thirteen (25%) CD patients were AEM negative. None were IgA deficient. Three AEM-negative CD patients had a raised IgA anti-tTG and IgA AGA. IgG AGA was raised in 10 AEM-negative CD patients, but also in 14/65 (22%) of controls. In conclusion, AEM-negative CD is common and detection is only modestly enhanced by testing for IgA anti-tTG antibodies. Duodenal biopsy is still recommended for the accurate diagnosis of CD.

KEY WORDS: anti-gliadin antibodies; anti-endomysium antibodies; anti-tissue transglutaminase antibodies; celiac disease.

The diagnosis of celiac disease (CD) is based on the 1989 European Society of Pediatric Gastroenterology and Nutrition criteria, which require characteristic small intestinal histology (1). The presence of two of three positive serological tests [anti-gliadin (AGA), anti-reticulin (ARA), and/or anti-endomysium (AEM)], are supportive of the diagnosis (1). Anti-endomysium antibodies are directed against the connective tissue surrounding smooth muscle fibers. They are detected using an immunofluorescence technique with positive samples giving a honeycomb

staining pattern in the connective tissue surrounding the myofibrils (2, 3). AEM antibodies are predominantly of the IgA-class (2), and the IgG-class AEM is not recommended for routine use, except in patients with IgA deficiency (4), as the specificity is lower than for the IgA assay (3). The reported sensitivity and specificity ranges of IgA AEM are 74–100% and 91–100% respectively (5–7).

IgA AEM fails to detect all gluten-sensitive individuals. The positive and negative predictive values of IgA AEM antibodies range from 79% to 100% and 95% to 100%, respectively (6–8). A positive AEM may depend on age, severity of the mucosal lesion (9, 10), possibly on the length of intestine involved (11), as well as on genetic factors (12). Assay sensitivity depends upon whether only seropositive patients are biopsied for confirmation or whether patients are biopsied on the basis of clinical suspicion.

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The enzyme tissue transglutaminase (tTG), has been identified as the predominant tissue autoantigen interacting with AEM antibodies in CD sera (13). Tissue transglutaminase is a calcium-dependent, cross-linking enzyme that forms proteolysis-resistant bonds between protein-bound glutamine and lysine residues (14). The enzyme is widely distributed in various tissues and body fluids including the gastrointestinal submucosa (15, 16). Gliadin's high glutamine content makes it a preferred substrate for tTG (17). An enzyme-linked immunosorbent assay (ELISA) method measuring IgA anti-tTG antibodies has potential advantages over the immunofluorescent AEM test in providing an objective, quantitative result.

Selective IgA deficiency is associated with a 10-fold increased risk of CD compared to the general population (18, 19). Relying on IgA-based serological testing to diagnose CD may produce misleading results in this group of patients. IgA-deficient patients have similar clinical presentations, histology, and response to treatment with a gluten-free diet (GFD) as patients with normal serum IgA (18, 19). IgG-class AGA, ARA, and/or AEM have been found to be useful in detecting these patients and in monitoring their response to a GFD (4, 18).

Even allowing for selective IgA deficiency, there is still a definite false-negative rate for the IgA AEM test (9, 10, 20). In this paper, we aim to prospectively investigate the role of the additional serological tests, in particular the IgA anti-tTG ELISA, in detecting IgA AEM-negative patients subsequently shown to have small intestinal biopsies consistent with CD and responding clinically to a GFD. To the best of our knowledge, this has not been previously reported.

MATERIALS AND METHODS

Patients. Fifty-three consecutive biopsy-proven CD patients (39 women, 14 men; age range 22–77 years, median 51 years) were prospectively studied between October 1997 and June 1999. Endoscopic duodenal biopsies were obtained from all patients using 37K rat-tooth, biopsy forceps (SB-37K-1, Olympus Endotherapy). All CD patients were taking a normal diet at the time of serum sampling and biopsy, and none of them were receiving immunosuppressants. Indications for investigation included diarrhea, weight loss, abdominal pain, anemia, and/or macrocytosis. Duodenal biopsies were performed on the basis of high clinical suspicion of CD and not only after the finding of positive serological results. Eight such patients had partial villous atrophy (PVA) and five patients had subtotal villous atrophy. Repeat biopsies following treatment with a gluten-free diet (GFD) were obtained in AEM-negative patients only. Control serum samples were obtained from 65 pa-

tients (46 women, 19 men; age range 17–90 years, median 45 years) investigated for suspected CD, but who subsequently were found to have normal duodenal mucosal histology.

Methods. All serum samples were analyzed for IgA-class AGA, AEM, and anti-tTG antibodies, as well as IgG-class AGA. Serum total IgA was measured in all untreated CD patients to screen for IgA deficiency. IgA deficiency was defined as a total serum IgA of less than 0.07 g/liter (reference range 1.01–4.23 g/liter). ELISA methods for determining AGA IgA and IgG were performed as previously described (21). The upper reference limits of IgA and IgG AGA were ≥ 30 units/ml and ≥ 45 units/ml, respectively. IgA/G/M-class R1 anti-reticulin antibodies were determined in the IgA AEM-negative patients only. Duodenal histology was reported by members of the pathology department as part of the routine national health service practice. Ethical approval was obtained from the Medicine and Oncology Subcommittee of Lothian Ethics in Research Committee.

IgA Anti-Endomysium Antibodies. Cryostat sections of human umbilical cord (8 μ m thick) were cut and stored at -20°C until use. Thawed sections fixed in acetone for 10 min at -20°C before transferring to chloroform for 30 min at room temperature. Each section was covered with ELISA diluent (0.9% saline, 0.05% Tween 20, 1% adult bovine serum) for 10 min and then drained. Test serum samples, diluted at 1/5 in ELISA diluent, were added to each section and incubated at room temperature for 60 minutes. A positive and negative control were included in each batch.

Sections were rinsed in phosphate-buffered saline (PBS, P-4417, Sigma) and then fluorescein isothiocyanate (FITC)-conjugated sheep anti-human IgA (Scottish Antibody Production Unit, Product No. S073-201) was added to each section and the slides incubated for 60 min. After washing in PBS and mounting in glycerin/gelatin, slides were examined by two independent readers (blinded to the patient details) at $\times 10$ and $\times 25$ magnifications using an immunofluorescence microscope (H500, Hund, Wetzlar). A positive result was recorded if the connective tissue surrounding the muscle cells fluoresced brightly in a honeycomb pattern. The AEM was reported as equivocal if the two experienced readers could not confidently discriminate the immunofluorescence pattern and did not represent disagreements between readers. The equivocal results have been grouped with the AEM-negative results for analysis in this paper.

IgA Anti-Tissue Transglutaminase Antibodies. Immulon 2 (Dynatech) plates were coated with 100 μ l/well of 10 μ g/ml solution of guinea pig liver transglutaminase (Sigma, T5398) diluted in a carbonate-bicarbonate coating buffer (0.05 M, pH 9.6, Sigma) and incubated overnight in a covered, moist box at 4°C . Plates were washed three times with ELISA wash (0.9% saline, 0.05% Tween 20) using a multireagent washer (Dynatech MRW, Dynatech Laboratories) and blocked with 250 μ l/well of blocking solution (PBS, 1% bovine serum albumin, 0.05% Tween 20) at room temperature for 2 hr. A serum reference standard from an untreated celiac patient was doubly diluted from 1/50 (in duplicate) to obtain a standard curve. High and low serum quality controls, and test serum samples were diluted in duplicate in diluent (PBS, 0.05% Tween 20) at 1/50 and

TABLE 1. RESULTS OF IgA AEM-NEGATIVE CELIAC DISEASE PATIENTS*

Patient	Age (yr)	IgA AGA (≤ 30 units/ml)	IgG AGA (≤ 45 units/ml)	IgA tTG (≤ 2950 units/ml)	Initial duodenal biopsy	Repeat duodenal biopsy after GFD
1 F	29	11	206	300	PVA	High IELs only
2 M	71	53	243	8440	PVA	NORMAL
3 F	43	17	181	830	Severe PVA	Severe PVA
4 F	52	23	75	300	STVA	Mild PVA
5 F	40	7	4	300	STVA	ND
6 F	77	29	146	410	PVA	PVA
7 F	52	5	8	940	PVA	NORMAL
8 F	70	590	315	6880†	STVA	ND
9 F	71	6	236	600	STVA	Mild PVA
10 F	63	13	97	300	PVA	NORMAL
11 M	40	12	11	300	STVA	PVA
12 F	37	46	86	8620	Severe PVA	Severe PVA
13 M	47	19	145	1150	PVA	Mild PVA

*Abnormal serological results are indicated in bold type. ND = not done; PVA = partial villous atrophy; STVA = subtotal villous atrophy; IELs = intraepithelial lymphocytes. Patients 3, 6, and 12 did not have histological improvement after intervals of 20, 13, and 10 months therapy with a GFD, respectively. Only patient three admitted to dietary noncompliance. The other two patients improved symptomatically following dietary therapy.

†The patient had an equivocal AEM result. The AEM was reported as equivocal if the two experienced readers could not confidently discriminate the immunofluorescence pattern.

1/100. Plates were incubated overnight at 4°C and then washed three times in ELISA wash.

One hundred microliters per well of goat anti-human IgA (α -chain specific) alkaline phosphatase conjugate (A-3063, Sigma), diluted at 1/800, was incubated at room temperature for 5 hr and then washed three times with ELISA wash. A *p*-nitrophenylphosphate (Sigma Product No. 104-105) substrate was added, and the color development was monitored by measuring the absorbance at 405 nm on an ELISA reader (DIAS microplate reader, Dynatech MR 5000, Dynex Technologies, Guernsey, UK). The absorbance end point for IgA tTG is when the top standard reaches 1.0. The test results were technically acceptable if the quality control anti-tTG IgA concentrations were within ± 2 SD of its mean values and the duplicate values differed by no more than 10%. The inter- and intraassay coefficients of variation were 9.1% and 11.7%, respectively. The normal upper reference limit for the IgA anti-tTG ELISA was obtained by testing the serum samples of 106 anonymous blood donors. A reference value of 2950 units/ml was calculated using a nonparametric percentile method (22), which excluded 95% of the blood donor subjects.

IgA Anti-Reticulin Antibodies. R1 anti-reticulin (ARA) antibodies were detected by an indirect immunofluorescent technique using a composite block of rat liver, kidney, and stomach. Sections 5 μ m thick were mounted onto glass slides and fixed in acetone for 5 min at room temperature. Test and positive control sera, diluted at 1/20 in PBS, were added to substrate wells and incubated at room temperature for 30 min. After washing in PBS, an IgA fluorescein-labeled anti-human globulin conjugate (Binding Site, Birmingham, UK) was added and incubated for 30 min. A mixed conjugate of IgG, A, and M from Atlantic Antibodies) was also used. After a further wash in PBS, slides were mounted in glycerol. A positive reticulin result was indicated by an apple green fluorescence at titers $\geq 1/20$.

Statistical Analysis. The sensitivities of the serological methods were calculated as the frequency of positive results

in biopsy-proven CD patients while on a normal diet. Assay specificities were calculated as the frequency of negative results in the 65 control patients. Positive and negative predictive values of each serological method (except for anti-reticulin antibodies) were calculated. The χ^2 was used to compare the frequency of symptoms and autoimmune diseases in AEM-negative and -positive CD patients. The Mann-Whitney test was used to compare the duration of symptoms, delay in diagnosis, as well as calcium and albumin concentrations of AEM-negative versus -positive CD patients.

RESULTS

Serology Results. Of the 53 untreated CD patients, 13 (25%) were negative for IgA AEM, 18 (34%) were negative for IgA anti-tTG, 19 (36%) were negative for IgA AGA, and 8 (15%) were negative for IgG AGA. Three CD patients were negative for all serological markers studied. Two female patients, ages 29 and 71 years, with low serum total IgA of 0.99 and 0.78 g/liter, respectively (reference range 1.01–4.23 g/liter), were both positive for IgG AGA only. None of the patients studied had selective IgA deficiency, and all were confirmed to be taking a normal gluten-containing diet at the time of serum sampling.

The 13 IgA AEM-negative CD patients ranged in age from 29 to 77 years; median 52 years (Table 1). Serum IgA anti-tTG antibody concentrations were raised in 3/13 AEM-negative CD patients, one of whom had dermatitis herpetiformis. The duodenal histology in two of these three patients showed PVA, and the other patient had STVA. The IgA AGA concentration was raised in the same three AEM-

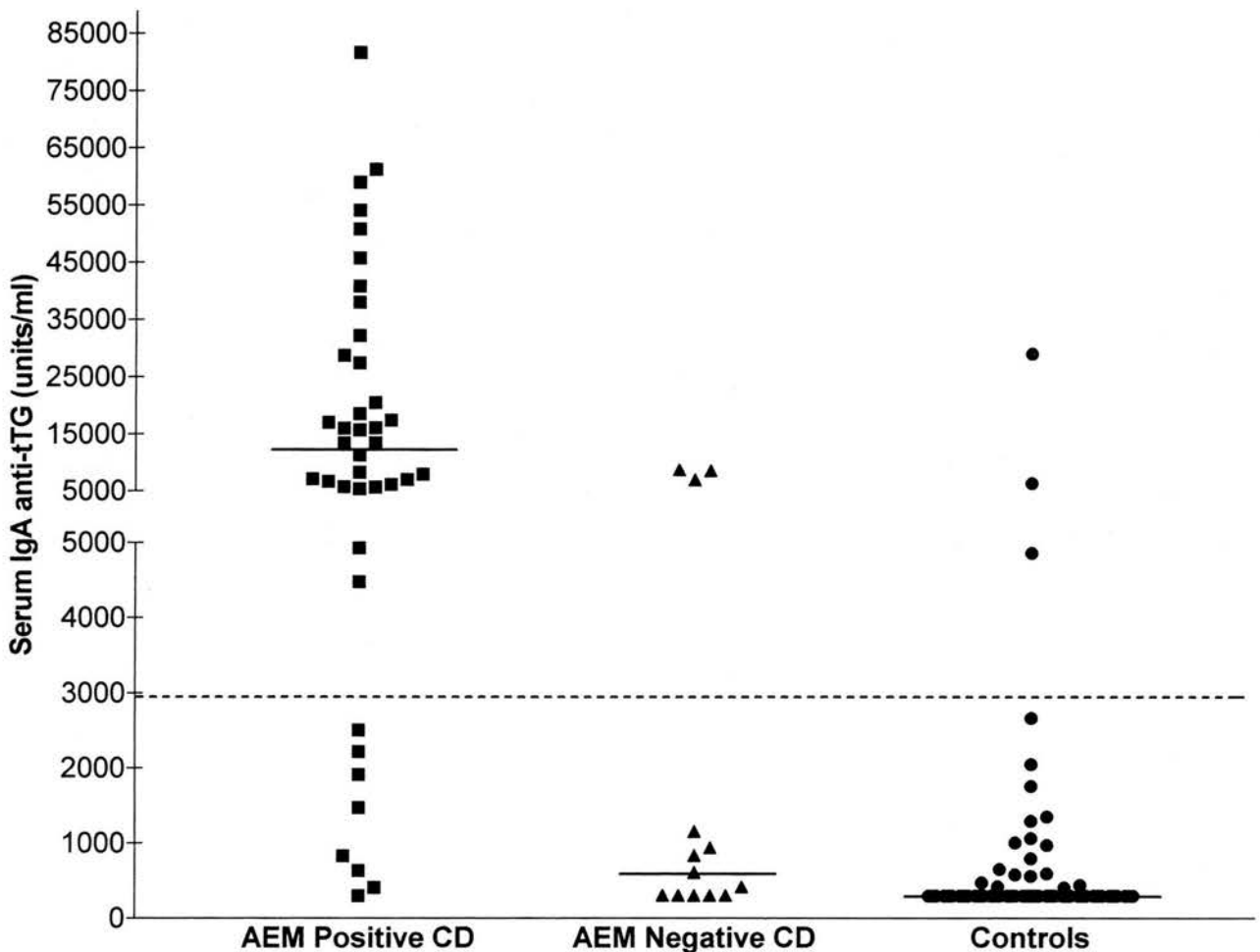


Fig 1. Serum IgA anti-tTG antibody concentrations in IgA AEM positive and negative celiac disease and disease control patients.

negative CD patients. The IgG AGA concentration was elevated in 10/13 AEM-negative CD patients. The initial duodenal histology in these 10 subjects showed PVA in seven patients and STVA in three patients. IgA/G/M ARA were negative in all IgA AEM-negative patients.

All of the 65 control patients underwent distal duodenal biopsy. All of these control subjects had a normal duodenal histology and a negative IgA AEM. Raised IgA anti-tTG antibody, IgA AGA, and IgG AGA concentrations were found, however, in 3 (5%), 10 (15%), and 14 (22%) control patients, respectively. The serum IgA anti-tTG antibody concentrations in AEM negative and positive CD patients, as well as controls are shown in Figure 1. The sensitivities, specificities, positive and negative predictive values of the serological tests are compared in Table 2.

Combination of Serological Tests. Serological detection of untreated CD was increased by using a

combination of tests (Table 3). In the 13 AEM CD-negative patients, the addition of IgA anti-tTG, IgA AGA, or IgG AGA would have detected 3, 3, and 10 untreated CD patients, respectively.

Repeat Duodenal Biopsies in IgA AEM-Negative CD Patients After Treatment with GFD. Repeat duodenal biopsies were performed in 11 of the AEM negative CD patients after a median duration of 10 months (range 4–20 months) of treatment with a GFD (Table 1). Histological appearances had markedly improved in eight patients, but remained unchanged in three patients. One of these three patients gave a clear history of dietary noncompliance. The other two compliant patients had been rebiopsied after an interval of 10 and 13 months of dietary therapy. Although there was no histological remission in these three IgA AEM-negative CD patients, all individuals reported a symptomatic improvement following gluten exclusion.

TABLE 2. SENSITIVITY AND SPECIFICITY OF SEROLOGICAL METHODS*

Serological test	Sensitivity (N = 53)	Specificity (N = 65)	Predictive Value	
			Positive	Negative
IgA AGA	64% (N = 34)	85% (N = 55)	77% (34/44)	74% (55/74)
IgG AGA	83% (N = 44)	78% (N = 51)	76% (44/58)	86% (51/59)
IgA AEM	75% (N = 40)	100% (N = 65)	100% (39/39)	83% (65/78)
IgA tTG	66% (N = 35)	95% (N = 62)	92% (35/38)	78% (62/80)

*IgA AEM is the most specific test, but IgG AGA is more sensitive in diagnosing celiac disease. The sensitivity and specificity of the IgA AEM test is higher than that of the IgA anti-tTG ELISA.

Repeat duodenal biopsies were not obtained in two patients, as one patient declined the appointment and the procedure was technically difficult in the other. Of note, the duodenal histology had markedly improved in two of the three CD patients in whom all serological tests were negative. The other patient was the individual who failed to attend for repeat biopsies.

Clinical Characteristics of IgA AEM-Negative CD Patients. Other investigations were frequently performed in the AEM-negative patients. Eight patients (seven women, one man, age range 29–77 years, median 50 years) had colonic investigations (four colonoscopies, three barium enemas, two flexible sigmoidoscopies). All of these examinations, including biopsies, were entirely normal except in three female patients. A 71-year-old had diverticular disease, a 63-year-old had proctitis due to collagenous colitis, and a 40-year-old had ileal atrophy which prompted duodenal biopsy. Small bowel contrast studies were performed in four patients (three women: one man; age range 40–77 years, median 56 years). Malabsorptive features were present in two patients and the other two were normal.

All of the AEM-negative patients reported gastrointestinal symptoms prompting investigation, but

there was no significant difference observed in the presenting features of AEM-negative and -positive CD patients. (Table 4) AEM-negative patients did, however, have significantly lower serum albumin concentrations (range 26–43 g/liter, median 38 g/liter) than AEM-positive patients (range 28–47 g/liter, median 40 g/liter) (Mann-Whitney test, $P = 0.03$). (Table 4) Autoimmune diseases were not seen more frequently in AEM-negative patients with two (15%) patients affected (one with hypothyroidism, one with collagenous colitis), than AEM-positive patients with four (10%) affected (two IDDM, one hyperparathyroidism, one Sicca syndrome) ($\chi^2 = 0.25$, $P = 0.616$).

The duration of symptoms in the AEM-negative patients (range 1–300 months; median 24 months) was not significantly longer than for AEM-positive patients (range 1–216 months; median 12 months) (Mann Whitney test, $P = 0.26$). The interval between gastroenterology assessment and diagnosis of CD in AEM-negative patients (range 1–72 months; median 2 months) was not significantly longer than

TABLE 3. SENSITIVITY OF SEROLOGICAL MARKERS WHEN USED IN COMBINATION*

Serological tests	Sensitivity	Specificity
IgA AEM	39/53 (74%)	65/65 (100%)
IgA AEM + IgA AGA	42/53 (79%)	56/65 (86%)
IgA AEM + IgG AGA	50/53 (94%)	51/65 (78%)
IgA AEM + IgA anti-tTG	42/53 (79%)	62/65 (95%)
IgA AEM + IgA AGA + IgG AGA	50/53 (94%)	44/65 (68%)

*The combination of the IgA AEM test with IgG AGA \pm IgA AGA produces the highest sensitivity in diagnosing celiac disease. The addition of IgA-class antibody tests to IgA AEM do not markedly enhance sensitivity. This is only achieved by the addition of IgG AGA.

TABLE 4. INDICATION FOR INVESTIGATION OF AEM-NEGATIVE CD PATIENTS

Presenting problem	AEM negative patients (N = 13)	AEM positive patients (N = 40)	Significance value (P)
Diarrhea	10 (77%)	28 (70%)	NS
Weight loss	8 (62%)	23 (58%)	NS
Abdominal pain	7 (54%)	18 (45%)	NS
Bloating	5 (38%)	18 (45%)	NS
Fatigue	9 (69%)	30 (75%)	NS
Anemia	6 (46%)	22 (55%)	NS
Hypoalbuminemia	2 (15%)	2 (5%)	0.03
Hypocalcemia	1 (7.6%)	2 (5%)	NS

*The clinical features of IgA AEM-negative celiac disease patients is not significantly different from AEM-positive patients with the exception of a low serum albumin which is observed more frequently in AEM-negative patients. NS = not significant.

for AEM-positive patients (range 1–48 months; median 1 month) (Mann Whitney, $P = 0.08$).

Of note, the longest delays in diagnosis of CD were seen in three AEM-negative female patients. One 40-year-old patient presented in 1984 with diarrhea and had a normal jejunal biopsy and small bowel contrast study, but raised markers of bile acid malabsorption. Worsening symptoms were investigated by colonoscopy, and she was found to have ileal atrophy. Subsequent endoscopic duodenal biopsies showed patchy STVA 60 months after initial presentation. Both IgA- and IgG-class serological markers were negative in this patient. This patient improved symptomatically following treatment with a gluten-free diet, but failed to attend for a repeat duodenal biopsy.

Another AEM-negative patient (age 43 years), presenting with iron-deficiency anemia, initially presented in 1993 when the diagnosis of CD was considered because of an elevated serum and jejunal aspirate IgA AGA; however, endoscopic duodenal biopsies showed “nonspecific changes” only. A jejunal biopsy failed to obtain sufficient tissue. The diagnosis of CD was finally made 72 months later (after several missed appointments) on repeat duodenal biopsies which showed severe PVA.

The last patient (aged 52 years) with diarrhea, weight loss, osteoporosis, and hypocalcaemia, was found to have negative IgA AGA and AEM antibodies five years earlier at another hospital, but duodenal biopsies were not performed. Duodenal biopsies obtained 60 months after her initial presentation showed STVA consistent with untreated CD. Repeat biopsies showed a good response to her GFD.

DISCUSSION

This study shows that 25% of newly diagnosed CD patients are negative for IgA AEM antibodies while consuming a normal gluten-containing diet. Others have also found a low IgA AEM sensitivity of 74–78% (6, 9). Indeed few studies have been able to reproduce the reported 100% sensitivity and specificity of IgA AEM (5, 8, 23). The sensitivities of the IgA-class antibody tests are reduced by unrecognized IgA deficiency and immunosuppressive therapy, which may produce false negative results (24); however, none of our newly diagnosed CD patients were receiving immune modifying drugs or had selective IgA deficiency.

The use of additional serological tests may help to identify some of these AEM-negative CD patients, as we could detect three of these patients by measuring

either the IgA anti-tTG or IgA AGA antibodies. ARA was of no added benefit as it did not detect any of these patients. IgG AGA concentrations were raised in 10 AEM-negative CD patients, but were also elevated in 14/65 (22%) control patients, compared to 10/65 (15%) for IgA AGA. Therefore IgG AGA increases detection of IgA AEM negative CD patients, but may not be suitable for use as a screening tool due to its lower specificity than IgA AGA. Combining IgG AGA with the more specific IgA AGA test may be the most appropriate compromise.

The IgA anti-tTG ELISA, using the guinea pig liver enzyme, has shown promising sensitivities and specificities in untreated CD ranging from 85 to 98.1% and 94 to 98% (25–29) respectively. However, few AEM-negative untreated CD patients were included in these studies: 10/136 (25), 1/106 (26), 1/27 (27), and 0/39 (29). The lower sensitivity of the IgA anti-tTG ELISA in this study of 66% is in part due to the inclusion of a large number of IgA AEM-negative CD patients. If these AEM-negative patients were excluded, the sensitivity of the IgA anti-tTG ELISA would improve to 82% (32/39). This approaches the 85% sensitivity quoted in a recent study that included AEM-positive CD patients only, and used a very similar IgA anti-tTG ELISA methodology (29). Unlike previous reports, this study does not show that the sensitivities of IgA AEM (30) and IgA anti-tTG (31) depend on the severity of the histological lesion as 5/13 AEM negative and 10/18 anti-tTG negative patients had STVA on duodenal biopsy.

AEM-negative CD patients present a challenge to both diagnosis and follow-up. AEM-negative and -positive patients have similar clinical presentations, frequency of autoimmune diseases, duration of symptoms, and calcium concentrations, but AEM-negative patients have significantly lower serum albumin concentrations than AEM-positive patients. In support of the diagnosis of CD, all of the AEM-negative CD patients responded symptomatically to gluten withdrawal, and a histological improvement was documented in 8 of the 11 patients who underwent repeat duodenal biopsy. In one of the three patients in whom there was no histological response to dietary gluten exclusion, there was a clear history of dietary non-compliance. The presence of symptomatic, but not histological improvement in two compliant patients after a period of up to 13 months therapy with a GFD, does not exclude the diagnosis of CD in these subjects, as it is known that the histological recovery may still be incomplete after two to four years (32).

The absence of a humoral response to tTG or to

AEM in some of our untreated CD patients casts doubt on the role of autoantibodies in the pathogenesis of CD. It has recently been reported that serum anti-tTG antibodies interfere with cellular differentiation and may contribute directly to mucosal flattening (33). It does seem unlikely however, that antibodies contribute directly to the mucosal atrophy as CD is common in selective IgA deficiency (18, 19) and may occur in severe hypogammaglobulinaemia (34). As tTG has been shown to be important in wound healing, it is possible that anti-tTG antibodies may contribute to the pathogenesis of CD by preventing tTG from performing its physiological functions, for example, in the restitution of mucosal integrity after injury.

The inclusion of AEM-negative patients in CD studies is important as the reporting of predominantly AEM-positive patients distorts the performance of serological tests. The high prevalence of AEM-negative patients in these consecutively diagnosed CD patients shows the importance of maintaining intestinal biopsy as the "gold standard" for the diagnosis of CD. The varying presentations of CD require a high degree of clinical suspicion and alertness to make an accurate diagnosis. The consequences of a delay in the diagnosis of CD are significant, as there is an increased frequency of lymphoma in untreated CD to the order of 40- to 100-fold (35, 36), and also of small bowel adenocarcinomas and esophageal and pharyngeal squamous carcinomas (37). Small intestinal biopsy should be performed in seronegative patients with a clinical suspicion of CD and should be repeated following treatment with a gluten-free diet.

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